

**WOUND RESPONSES OF *EUCALYPTUS GLOBULUS* AND
E. NITENS; ANATOMY AND CHEMISTRY**

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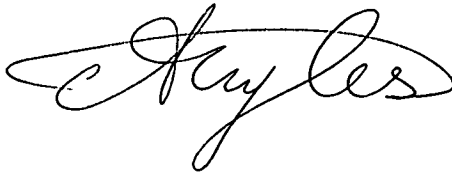
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University of Tasmania, 2003.

DECLARATIONS

This thesis does not contain any material, which has been accepted, for a degree or diploma by the University of Tasmania or any other institution. To the best of my knowledge, this thesis contains no material previously published or written by another person except where due acknowledgement is made

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ABSTRACT

The anatomical and chemical host responses of two economically important *Eucalyptus* species grown in southern Australia (*E. globulus* and *E. nitens*) to different wounding treatments (mechanical, chemical and biological) were investigated. Initially, the project primarily focused on the role of kino veins as barrier zones in eucalypt tree defence but then later also included the characterization of the new tissue formed subsequent to wounding, an area of study previously never been studied in any eucalypt species.

The developmental anatomy and structure of kino veins was examined in three juvenile *Eucalyptus* species (*E. nitens*, *E. globulus* and *E. obliqua*) to treatment with an ethylene-releasing compound, 2-chloroethyl phosphonic acid (CEPA). Unlike *E. globulus* and *E. obliqua*, *E. nitens* failed to produce kino veins in response to the hormone treatment, confirming anecdotal evidence that this species does not readily form kino veins. During this study, histochemical stains including *p*-dimethylaminocinnamaldehyde (DMACA) and Coomassie brilliant blue G-250 (BBG-250) were refined to allow the localization of condensed tannins and hydrolyzable tannins, respectively in wood sections.

The production of dark extractives (non-structural components of wood and bark) was regularly observed as part of the wound response in both the phloem and/or xylem tissue and regardless of the cause of injury. The various wound treatments examined included drill wounding with fungal inoculations, dry ice wounding, natural infection to stem canker (*Cytospora* sp.) and prune wounding. Analysis of dark extractives extracted from the new tissue formed after wounding using gas chromatography combined with mass spectrometry (GC-MS), high performance liquid chromatography (HPLC-UV) coupled to negative ion electrospray mass spectrometry (ESI-MS) revealed that a complex range of secondary metabolites was present, including hydrolyzable tannins, proanthocyanidins, flavonone glycoside, stilbene glycosides, formylated phloroglucinol compounds (FPCs) and volatile terpenes. These compounds were either undetectable in healthy tissue or else present at

significantly lower concentrations than in wound-associated tissue. In particular, the detection of FPCs in the wound-associated wood was a novel finding, hitherto detected in the leaves of various eucalypt species. We suggest that the diverse range of secondary metabolites detected in the wound-associated tissue may have a multi-functional role in relation to tree wound repair and defence. Crude wound wood extracts were shown to possess *in vitro* antimicrobial activity against decay fungi and gram-positive bacteria as well as *in vitro* antioxidant activity.

We detail the first reported case of traumatic oil glands induced by wounding in eucalypts. Histological examinations revealed the new phloem tissue formed in the two years following green pruning in 5-year-old *E. globulus* to be largely composed of secretory cavities similar in appearance to oil glands. Subsequent analysis of extracts by GC-MS confirmed the presence of volatile terpenes and phenols. The total oil content determined for wound-associated phloem extracts was significantly higher (>50 times) than for healthy stem phloem extracts.

Based on these chemical and morphological findings, we propose a reassessment of the importance of wound wood in tree compartmentalization response for *E. globulus* and *E. nitens*, particularly in comparison with other woody tree species.

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‘Knowledge is power’

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‘Doubt increases with knowledge’



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PREFACE

This PhD thesis is composed of 7 papers (which have been either published or submitted for publication to refereed international journals) as well as three additional chapters. To

improve the reading of the thesis, the following changes have been made including;

- References have been aggregated to single list of references at the end of the thesis,
- Acknowledgements for each publication have been detailed separately (see below),
- Figures and tables have been renumbered according to the chapter,
- Additional details that were not originally included in the papers but considered worthy contributions to the thesis have been added as appendices where required.

Publications arising from this project are as follows:

Refereed journal papers

CHAPTER 2

Eyles, A. and Mohammed, C. 2002. Comparison of CEPA (2-chloroethyl phosphonic acid) induced responses in juvenile *Eucalyptus nitens*, *E. globulus* and *E. obliqua*: a histochemical and anatomical study. International Association of Wood Anatomists (IAWA) Journal. 23(4): 419-430.

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CHAPTER 3

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CHAPTER 5

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CHAPTER 6

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CHAPTER 7

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CHAPTER 8

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Refereed conference proceedings

1. Mohammed, C.L., Barry, K.M., Battaglia, M., Beadle, C.L., **Eyles, A.**, Mollon, A., and Pinkard, E.A. 2000. Pruning-associated stem defects in plantation *E. nitens* and *E. globulus* grown for sawlog and veneer in Tasmania. Eds L Henderson, G Waugh,

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Unrefereed conference proceedings

1. **Eyles, A.**, Davies, N.W., Mohammed, C., Mitsunaga, T. and Mihara, R. 2003. *Eucalyptus* wound wood extractives show antimicrobial and antioxidant activity. VIII International Congress of Plant Pathology. Poster. 2-7 February, Christchurch, New Zealand. (Appendix 10.1).
2. Mohammed, C., Wardlaw, T.J., Barry, K., **Eyles, A.**, Wiseman, D., Beadle, C., Battaglia, M., Pinkard E. and Kube, P. An interdisciplinary approach to the study and management of stem defect in eucalypts. Contributed Symposia. 2-7 February, Christchurch, New Zealand.
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5. Mohammed, C., Barry, K.M., **Eyles, A.**, Harrison, K. and Hall, M. 2002. An overview of wound response and antimicrobial defence in eucalypts VII International Mycological Congress. Contributed Symposia. Oslo, 11-17 August. Norway.

6. Eyles, A., Mohammed, C.L. and Davies, N.W. 2001. Eucalypt tree responses to wounding. Invited Presentation at 'CRC Annual Meeting' 29 October – 31 October. Caloundra, Queensland.

7. Eyles, A., Davies, N.W. and Mohammed, C.L. 2001. Anatomy and chemistry of wound tissue in *Eucalyptus nitens* and *E. globulus*. Poster at CRC-SPF Annual Meeting' 29 - 31 October. Caloundra, Queensland. (Appendix 10.1).

8. Eyles, A. 2000. Expression of kino: does it have a role in wound defence? *Thinning and Pruning Workshop*. Presentation. May 3-5. Gympie, Queensland, Australia.

9. Mohammed, C.L., Barry, K.M., Battaglia, M., Beadle, C.L., Eyles, A., de Little, D.W., Milgate, A.W., Mollon, A., Smethurst, P.J., Vaillancourt, R.E., Yuan, Z.Q. 1999. Research strategies for disease management in eucalypt plantations in Tasmania, Australia. In '12th Biennial Australasian Plant Pathology Society Conference.

10. Mohammed, C.L., Barry, K.M., Battaglia, M., Beadle, C.L., Eyles, A., Hall, M.F., Milgate, A.W., Mollon, A., Smethurst, P.J., Vaillancourt, R.E., Yuan, Z.Q. 1999. Strategies for disease management in Eucalyptus plantations in Tasmania, Australia. In '18th Biennial Conference of the Institute of Foresters of Australia'. (Eds RC Ellis and PJ Smethurst) p. 188. (IFA Tasmanian Division: Hobart, Tasmania)

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CHAPTER 1: GENERAL INTRODUCTION**1.1 EUCALYPTS AS IMPORTANT HARDWOOD SPECIES FOR WOOD PRODUCTION**

Eucalyptus has valuable wood and pulp characteristics and many useful silvicultural properties including high growth rates and adaptability to a wide range of soils and climates (Turnball, 2001). Of the estimated 187 million hectares of forest plantations world wide in 2000, twenty percent are *Pinus* plantations, ten percent are *Eucalyptus* plantations and the rest include minor species (Anon, 2001).

In Australia, there presently exists a total of 1.57 million hectares of plantation forest, comprising of approximately 1 million hectares of softwood pines and about 500 000 hectares of hardwood eucalypts. This figure is projected to double by 2020, with most new plantations to be established on previously cleared agricultural land (Gerrand *et al.*, 2003). *E. globulus* (Labill) and *E. nitens* (Maiden) are the two predominant hardwood plantation species grown in southern Australia (Tibbits *et al.*, 1997). Both species belong to the family Myrtaceae and are well recognised for their fast growth rates. Furthermore, the intensive management that occurs in plantations can result in even faster growth rates, up to three times faster than those measured in native forests (Dargavel, 1990). In Tasmania, *E. nitens* is the preferred species for high quality timber production due to good growth rates on a variety of sites, frost tolerance, good form and wood quality suitable for pulp, sawn timber and veneer (Gerrand, *et al.*, 1997). The characteristics of each species are outlined in Table 1.

Table 1. Silvicultural properties of *E. globulus* and *E. nitens*

Characteristics	Species	
	<i>E. globulus</i> *	<i>E. nitens</i> **
Natural Distribution	Coastal and near-coastal areas of eastern and southeastern Tasmania, Bass Strait islands, Otway Ranges and in southern Gippsland in Victoria	Great Dividing range from New South Wales to Victoria
Altitudes (m)	0-650	600-1600
Rainfall (mm)	500-2000	750-1750
Frost tolerance	Nil	High
Drought tolerance	High	Low
Commercial plantations in Australia	Southern and Western Australia.	Tasmania and cooler areas of Victoria

* From Tibbits *et al.*, 1997.

**From Boland *et al.*, 1984 and Pederick, 1979.

Over 120 000 hectares of hardwood plantation (mainly eucalypts) are currently present in Tasmania. Most plantations in Australia are grown for pulpwood. However, in Tasmania, approximately 30 000 hectares of both *Eucalyptus nitens* and *Eucalyptus globulus* have been planted for the provision of sawn timber and veneer products on rotations of 30-plus years (C. Beadle, personal communication).

1.2 INTENSIVE MANAGEMENT OF *EUCALYPTUS* IN AUSTRALIA.

Until recently, the Australian eucalypt forestry industry supplied a range of wood products by logging native forests. Increasingly native forest is being set aside under reserve and logging is prohibited. The eucalypt industry has had to adopt systems of sustainable wood production. Timber is extracted from intensively managed native forests and plantations. In order to produce logs of sufficient quality, regenerated forest trees must be thinned and plantations, pruned and thinned (Dudzinski *et al.*, 1992; Gerrand *et al.*, 1997).

1.2.1 Green pruning

When grown in natural forest conditions, most eucalypt species efficiently shed dead lower branches (Shepherd, 1986). However, when grown in plantations where fast growth is promoted, eucalypt species retain rather than shed dead branches resulting in the development of unwanted large knotty cores and loose knots in processed timber (Wardlaw, 1999, Mohammed *et al.*, 2000). Industry has attempted to overcome this obstacle by 'green pruning' i.e. the removal of live branches. Green pruning aims to minimise the diameter of the knotty core as well as restrict the development of loose knots, thereby maximising the amount of clearwood recovered for select grade material (Pinkard, 1999) (Fig. 1.1). Green pruning is generally carried out at the time of most rapid growth so as to minimize the effect on growth and ensure fast occlusion of the branch stubs (Pinkard and Beadle, 1998).

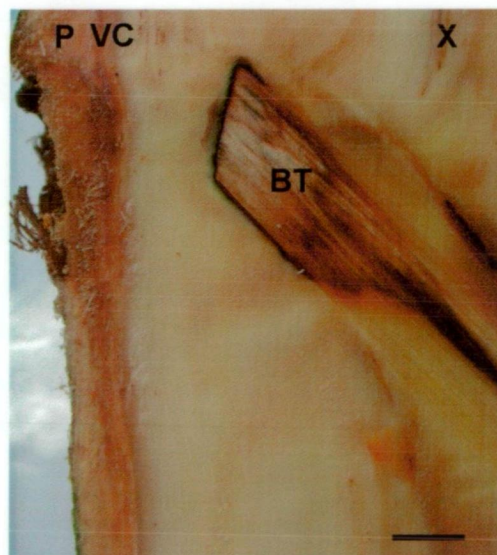


Fig. 1.1 Radial longitudinal section of cleanly occluded branch 2 years after green pruning in 5-year-old *E. nitens*. BT = branch trace, P = phloem, VC = vascular cambium, X = xylem. Bar ~ 1cm.

1.2.2. Thinning

Thinning, which involves the selective removal of individual tree within a stand, is the key management option used for improving yield of high quality timber suitable for sawn timber and veneer production without the need of long rotation periods (White and Kile, 1991).

Thinning aims to maximise the diameter growth of the remaining trees by preventing the remaining trees from experiencing intra-specific competition. In this way, the characteristic

rapid early growth rate of *Eucalyptus* species can be maintained by the selected final crop trees (Medhurst, 2000).

1.2.3 Consequences of pruning and thinning

One of the potential consequences of thinning and pruning operations is mechanical wounding, which may result in an increased incidence of defect and decay (Vasilias, 2001). Clearly, wounding into the xylem provides a major route for the entry of decay-causing fungi into living trees. The few studies that have looked at the impact of pruning on the incidence of decay have identified the infection of pruning wounds as a major issue determining wood quality in eucalypts (Gadgil and Bawden 1981; Wardlaw, 1996; Gerrand et al., 1997; Mohammed *et al.*, 1998; Pinkard and Beadle, 1998; Wardlaw and Neilsen, 1999). They found the occurrence of stem decay associated with pruning was high compared with unpruned trees and the risk of decay was often highest on sites with the fastest tree growth rates. The risk of decay was also positively correlated with increasing branch size (Pinkard and Beadle, 1998).

Another possible problem arising from the mechanical damage incurred during thinning operations is that of kino vein formation (Fig. 1.2). Kino veins and/or pockets are characteristic defects of some *Eucalyptus* species and have long been recognised as sources of degrade in eucalypt timber. The exact cause of kino vein formation is still not clearly understood but it has been generally associated with a number of stresses that apparently damage but not kill the vascular cambium. These stresses include branch shed, damage by biological agents (e.g. insects and fungi), physiological stress, fire and mechanical damage (Jacobs, 1986). Within a tree defence framework, kino veins are generally considered to play a role in wound defence by acting as a 'barrier zone' (Wilkes, 1986; Hillis, 1987). A barrier zone is broadly defined as a protective tissue, which forms in response to infection as well as to mechanical wounding (Tippett and Shigo, 1981). Anecdotal evidence suggests that in intensively managed *E. globulus* plantations grown for sawlog production, there exists a

potential problem of sawlog downgrade due to kino vein incidence (Gerrand *et al.*, 1997, C. Mohammed, personal communication).



Fig. 1.2 Radial longitudinal section of kino veins (arrow) included in the xylem of 9-year-old *E. globulus*. P = phloem, VC = vascular cambium, X = xylem. Bar ~ 1 cm.

1.3 EUCALYPT WOUND RESPONSE

Fundamental research related to wound response of *Eucalyptus* species is still rudimentary. A better understanding of eucalypt tree defence may provide much needed background knowledge of tree resistance mechanisms as well as offer a sound basis for developing improved silvicultural practices. Most of our knowledge concerning the mechanisms of tree resistance has been largely derived from studies involving conifers and northern hardwood temperate trees including beech (*Fagus*), birch (*Betula*), oak (*Quercus*) and maple (*Acer*). While it is reasonable to assume, *a priori*, that the defence mechanisms operating in eucalypt species are likely to be similar to other species, the relatively few detailed studies of defence in northern hardwood species has indicated significant variation in mechanisms (Pearce, 2000). More importantly, recent chemical and morphological studies investigating reaction zones of *E. nitens* showed the responses were different in comparison with other angiosperms previously studied (Barry *et al.*, 2000 and 2001).

1.4 AIMS OF THIS RESEARCH

Initially, the objectives of this project were to investigate the factors influencing the incidence of kino veins in eucalypt plantations. This investigation led me to characterize the new tissue formed subsequent to wounding; an area of study previously never conducted in any eucalypt species. The objectives of the thesis can be summarized as follows:

- To carry out a chronological study on the development of kino vein formation in *E. nitens* and *E. globulus* with application of an ethylene-releasing compound, CEPA (2-chloroethyl phosphonic acid).
- To compare the incidence of kino formation of *E. nitens* and *E. globulus* when exposed to a variety of treatments including fungal inoculations and mechanical damage.
- To analyze the chemistry of extractives from the new wood and bark tissue produced after wounding and/or fungal infection with a) high performance liquid chromatography (HPLC-UV) b) HPLC coupled with negative ion electrospray ionisation mass spectrometry (HPLC-ESI-MS) and c) gas chromatography combined with mass spectrometry (GC-MS).
- To investigate the biological activity of dark extractives in wound defence by conducting *in vitro* antimicrobial bioassays and *in vitro* antioxidant assays.

CHAPTER 2

Comparison of CEPA (2-chloroethyl phosphonic acid) induced responses in juvenile *Eucalyptus nitens*, *E. globulus* and *E. obliqua*: a histochemical and anatomical study

Abstract

Kino veins and/or pockets are a characteristic defect of some *Eucalyptus* species and have long been recognised as sources of degrade in eucalypt timber. In a comparative study, the course of both histochemical and anatomical responses of three juvenile *Eucalyptus* species (*E. nitens*, *E. globulus* and *E. obliqua*) to treatment with an ethylene-releasing compound, 2-chloroethyl phosphonic acid (CEPA), were examined. Kino veins were induced in both *E. obliqua* and *E. globulus*. The growth regulator treatment failed to induce kino vein formation in *E. nitens*. The traumatic parenchyma (that later differentiated into kino veins) was not always derived from the initials of the vascular cambium as reported in previous studies in *E. globulus*. Instead, the traumatic parenchyma appeared to be produced from the meristematic activity of the inner phloem parenchyma. In addition, kino veins found in 2 to 3-year-old *E. globulus* were mostly included in the phloem and not in the xylem as generally observed in mature *E. globulus*.

2.1 INTRODUCTION

Eucalyptus globulus and *E. nitens* are widely planted in Tasmania for pulp and sawlog (Beadle and Mohammed, 1999). In intensively managed *E. globulus* plantations, there exists a potential problem of sawlog downgrade due to kino vein incidence (Gerrand *et al.*, 1997). Kino veins and/or pockets are a characteristic industrial defect of some *Eucalyptus* spp. arising from damaged cambial regions (Bamber, 1985). Most authors agree that kino veins generally play a role in wound defence as a tree resistance mechanism by acting as a 'barrier zone' (Hillis, 1987). A barrier zone is broadly defined as a protective tissue, which forms in response to infection as well as to mechanical wounding (Tippett and Shigo, 1981) and is characterized by a great amount of axial parenchyma and few conducting elements (Torelli *et al.*, 1994).

The plant growth regulator, ethylene is well known to play a key role in the formation of tree exudates (Hillis, 1975, 1987). The exogenous application of ethylene to induce or enhance

secretion of tree exudates has been well documented e.g. latex from *Hevea* species, gum from *Prunus* species and resin from *Pinus* species (Hillis, 1987). Similarly, application of Ethrel onto bark has been used to induce kino veins in the seedlings of several juvenile *Eucalyptus* species (Dowden and Foster, 1973). Ethrel is a commercial preparation of 2-chloroethyl phosphonic acid (CEPA), an ethylene-releasing compound. The developmental anatomy and structure of kino veins induced by either mechanical or chemical wounding has been studied in detail in only a few eucalypt species, e.g. *E. obliqua* (Skene, 1965) and *E. radiata* (Dowden and Foster, 1973). The developmental anatomy and structure of kino veins in *E. nitens* and *E. globulus* has not been previously studied. The present study reports the histochemical and anatomical responses of 2-3-year-old potted trees of three commercially important *Eucalyptus* species (*E. globulus*, *E. obliqua* and *E. nitens*) treated with CEPA.

2.2 MATERIALS AND METHODS

2.2.1 Induction of kino veins with CEPA

Six trees each of *E. globulus*, *E. obliqua* and *E. nitens* aged 2 and 3 years of age and approximately 5-7 cm in diameter were used. Trees were grown outside in a commercial soil mix and subject to regular application of liquid fertilizer and daily watering. Each tree was injected with a total of 0.3 ml of 1 % CEPA aqueous solution (2-chloroethyl-phosphonic acid, SIGMA) at 3 replicate points along the main stem (20, 40 and 60 cm above soil level). In addition, 0.3 ml of distilled water was also injected on the same stem at 80 cm above soil level as a control. A fine needle (Luer Hub, 24G x 1 In, Aldrich) was inserted into the phloem until the needle tip encountered the much harder xylem tissue. The CEPA and the water were then applied in a number of small increments to allow the tissue to absorb the solution. The experiment commenced in late spring. Harvesting of 1 cm³ wood samples, that included the needle site, occurred after 7, 14, 21, 28, 35 and 42 days. The experiment was conducted in late spring of 1999 and one year later.

2.2.2 Light microscopy

Wood samples were fixed in FAA (formaldehyde: acetic acid: 50% ethanol; 5:5:90) for a minimum of 24 h at 4°C, then dehydrated with an ethanol series (50%, 70%, 95%, 3 x 100%), infiltrated in L. R. White acrylic resin (ProSciTech, Brisbane) and polymerized for 8-10 h at 60°C. The embedded samples were sectioned at approximately 10-12 µm thickness with a sledge microtome. Additional sections of 5-10 µm thickness were obtained with a rotary microtome (Leitz 1516) fitted with a glass knife. Transverse and longitudinal sections were dried onto glass slides, stained with a 1 % aqueous solution of toluidine blue and permanently mounted in Cytoseal (Proscitech, Brisbane). Tissue structure and cell changes were noted. Photographs were taken with colour film (tungsten) on a Zeiss Axioskop photomicroscope fitted with a Zeiss MC 100 camera.

2.2.3 Staining methods

Cell wall histochemistry

Lignin presence was examined using the phloroglucinol-HCl test as described by Jensen (1962). Suberin (wall-bound lipid polymers) was stained with Sudan black B (Jensen 1962) in 70 % ethanol. In addition, suberin was detected with a Zeiss Axiovert fluorescence microscope using a mercury lamp. The autofluorescence of lignin was quenched with 1 % phloroglucinol-HCl as outlined in Biggs (1984).

Localization of proanthocyanidins

Kino veins produce a red-brown aqueous solution of polyphenolic compounds called kino. The main constituents of kino are mostly polyphenolic compounds, in particular, polymeric proanthocyanidins (syn. condensed tannins) (Hillis and Yazaki, 1975). A number of histochemical stains are commonly used for the localization of polyphenols e.g. incubation with the nitroso reagent or the ferric chloride test (Jensen, 1962). However, these methods are non-specific and selective staining of different tannins is not possible. In this study, two alternative methods were tested for the specific localization of proanthocyanidins within plant.

A) Monomeric flavan-3-ols as well as the corresponding oligomeric proanthocyanidins were detected with *p*-dimethylaminocinnamaldehyde (DMACA) (modified from Gutmann, 1993). The embedded sections were placed on glass slides, covered with the staining solution (2 mL of 0.1 % DMACA dissolved in 3 M sulphuric acid in 1-butanol) and then heated for around 10 s on a hot plate. A green/blue-coloured product was observed in the presence of proanthocyanidins. This method provided good results and was less time consuming than the method outlined by Gutmann (1993).

B) Detection of hydrolyzable tannins was carried out with the Bradford protein-binding dye, Coomassie brilliant blue G-250 (BBG-250). In a chemical assay, Kilkowski and Gross (1999) reported this dye to react specifically with hydrolyzable tannin standards to form intensively blue-coloured complexes. With this information in mind, a simple method was developed to localize the hydrolyzable tannins *in situ* with BBG-250. To effect this, the reactants were dissolved in water to avoid interference caused by organic solvents (as suggested by Kilkowski and Gross, 1999), applied to fresh sections for 10 minutes and then rinsed with water.

2.3 RESULTS AND DISCUSSION

2.4.1 Initial response to CEPA treatment

In all three species studied, the initial response to the CEPA treatment involved 'swelling' of the ray parenchyma located within the vascular cambium. The ray parenchyma appeared to undergo anticlinal divisions giving rise to bi - multi-seriate ray parenchyma (Fig. 2.1). After 'ray swell,' the ray parenchyma in the mature xylem as well as in the adjacent healthy cambial zone proliferated extensively such that the ray cell derivatives completely filled the injection wound gap (Fig. 2.2).

This initial wound response was similar to the wound healing response observed in other tree species such as conifers (Kuroda and Shimaji, 1984) and other hardwood species (Lev-Yadun and Aloni, 1995). The differentiation of ray parenchyma is an important process in

wound regeneration as callus produced from ray cells then has the potential to develop into a new cambium (Kozłowski and Pallardy, 1997). In addition, ray parenchyma cells are responsible for the synthesis of various phenolic compounds (Shortle 1979) and are the source of tyloses and polysaccharide deposits in vessels (Chattaway, 1949).

2.3.2 Response of *E. globulus* and *E. obliqua* to CEPA treatment

Macroscopic observations

Kino veins were induced in juvenile *E. globulus* and *E. obliqua* by the CEPA treatment (Table 2.1). The rate of kino vein development observed for *E. globulus* was generally slower compared to that for *E. obliqua*. Kino was observed to exude onto the bark surface after approximately 14 days in *E. obliqua* and 28 days in *E. globulus*. The kino was initially a sticky straw-coloured exudate that upon exposure to air polymerized to become brittle, assuming a cherry red to a brown/black colour (Appendix 2.1).

Table 2.1. Response of *E. globulus*, *E. obliqua* and *E. nitens* to treatments

Species	% kino veins induced	
	CEPA treatment	Control treatment
<i>E. globulus</i>	100	0
<i>E. obliqua</i>	100	30
<i>E. nitens</i>	0	0

Fig. 2.1-2. Response of ray parenchyma to injection wound.

2.1: Transverse section showing swelling of ray parenchyma located within the cambial zone (*E. globulus*). Bar = 75 μ m.

2.2: Transverse sections showing callus-like proliferation of ray cells (arrow) (*E. obliqua*).
Bar = 100 μ m.

CZ= cambial zone, IW = initial wound, RP= ray parenchyma, RS= ray swell, X = xylem.

Fig. 2.3. Radial longitudinal sections of kino vein development in the xylem of *E. obliqua* and *E. globulus* treated with CEPA.

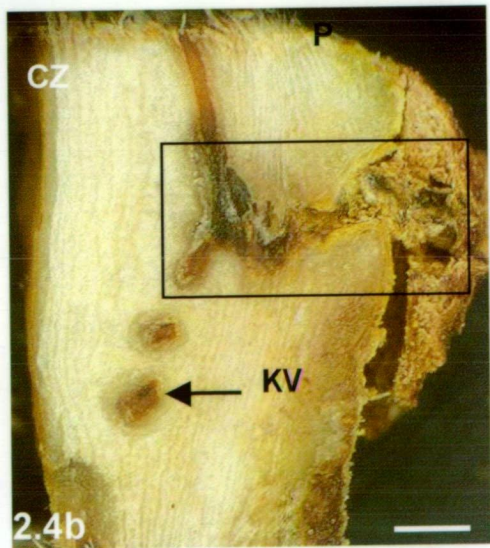
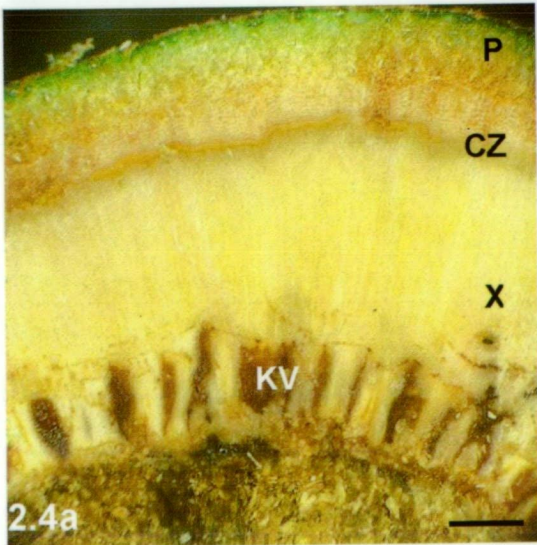
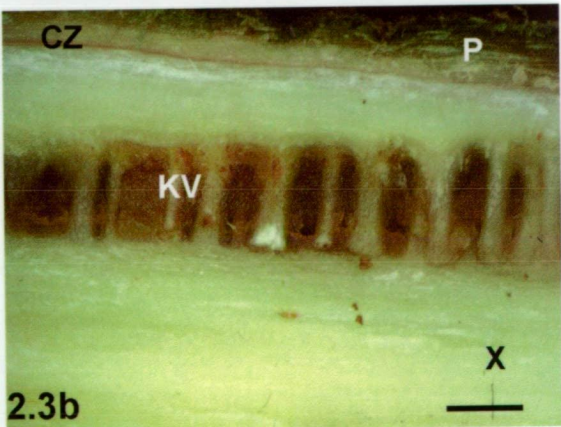
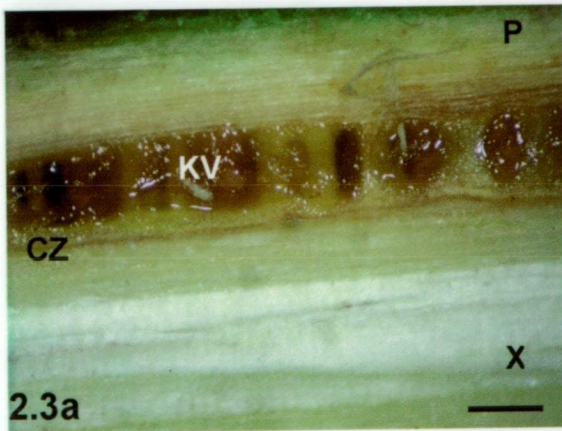
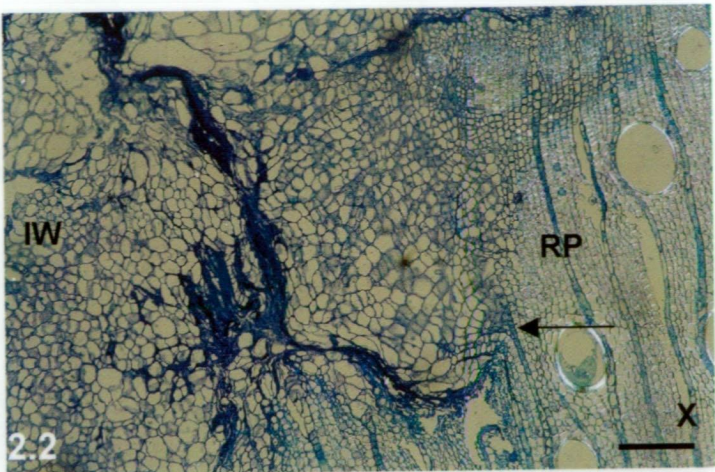
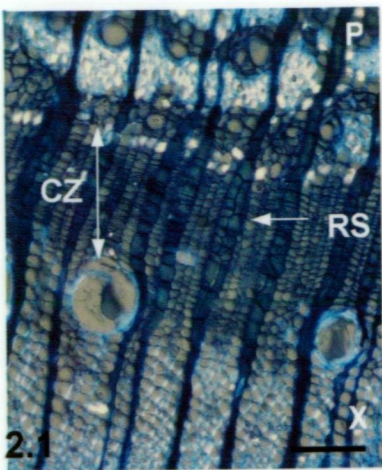
a: 35 days post-induction (*E. obliqua*).

b: 45 days post-induction (*E. globulus*). All bars ~ 0.5 cm.

CZ = cambial zone, KV = kino vein, P = phloem, X = xylem.

Fig. 2.4. A single juvenile *E. globulus* tree observed to produce kino vein in both xylem (a) and phloem (b). Location of insect borer found in square. All bars ~ 0.5 cm.

CZ = cambial zone, KV = kino vein, P = phloem, X = xylem.



The induced kino veins were mostly included in the phloem of *E. globulus* and in the xylem of *E. obliqua* (Fig. 2.3). Anatomical work carried out by Day (1959) found evidence to suggest that location of kino veins, i.e. whether kino veins are found in either the xylem or phloem, may be species dependent. Moreover, a survey of 93 *Eucalyptus* species (based on Pryor and Johnson's classification system, 1971) determined that species that exhibit kino veins in the xylem belong to the subgenera *Monocalyptus* and *Corymbia*. Species observed to develop kino veins in the phloem were all members of three sections of the subgenus *Symphyomyrtus* (Tippett, 1986). Therefore, according to Tippett, kino veins should be induced in xylem of *E. globulus* and *E. obliqua*. In this study, kino veins were always induced in the xylem of *E. obliqua*, however kino veins were mostly found in the phloem of *E. globulus*.

Juvenile *E. globulus* may have the potential to produce kino veins, which may be included in either phloem or xylem depending on the nature of the injury. For example, a single *E. globulus* tree exhibited kino veins in the phloem and in the xylem at two different locations along the bole (Fig. 2.4). The distinct but separate kino veins located in the phloem were associated with insect borer attack while the kino veins located in the xylem appeared to be associated with fungal decay. Alternatively, tree maturity may influence the location of kino veins. In nature, injury to eucalypt trees less than 4 to 5 years of age rarely results in kino vein formation (Dowden and Foster, 1973). Likewise, *Acacia senegal* needs to be 5-7 years of age before it is able to produce gum (Joseleau and Ullmann, 1990) suggesting that juvenile trees respond differently to wounding compared to mature trees. Evidence to support this alternative explanation can be found in this study. In the first year of the experiment when 2-year-old *E. globulus* trees were used, kino veins were always induced in the phloem following treatment with CEPA. But in the second year of the experiment, when 3-year-old *E. globulus* trees were used, it was difficult to determine from the anatomical sections whether the kino veins were induced in the phloem or xylem (Fig. 2.5d). In one area of the sections, the kino veins appeared to be included in the phloem while in another area of the same section; the kino veins appeared to be included in the xylem (Fig. 2.5d).

Fig. 2.5. Transverse (a, c-e) and radial longitudinal (b) sections showing kino vein development in *E. globulus*.

a: Control. Bar = 200 μm .

b: Expanded thin-walled traumatic parenchyma (*) observed 21 days post-induction.

Prominent nuclei in the newly differentiated cells are located in the square. Bar = 150 μm .

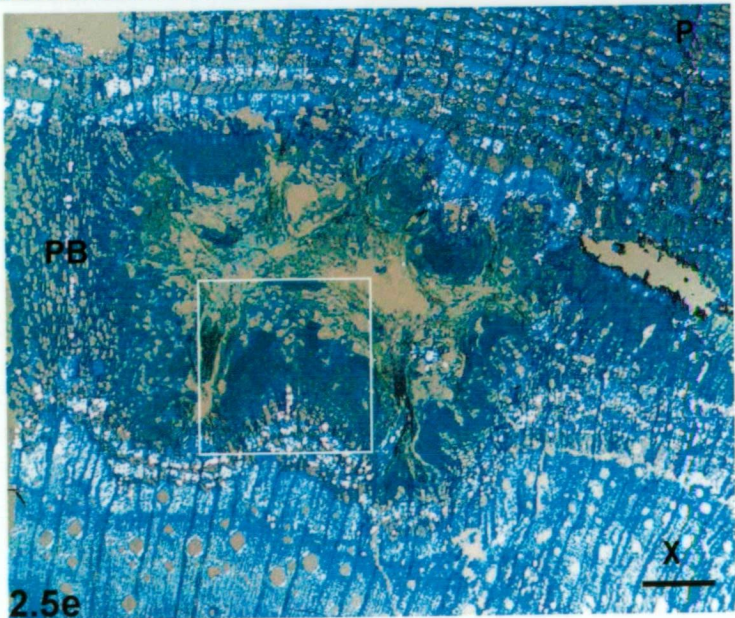
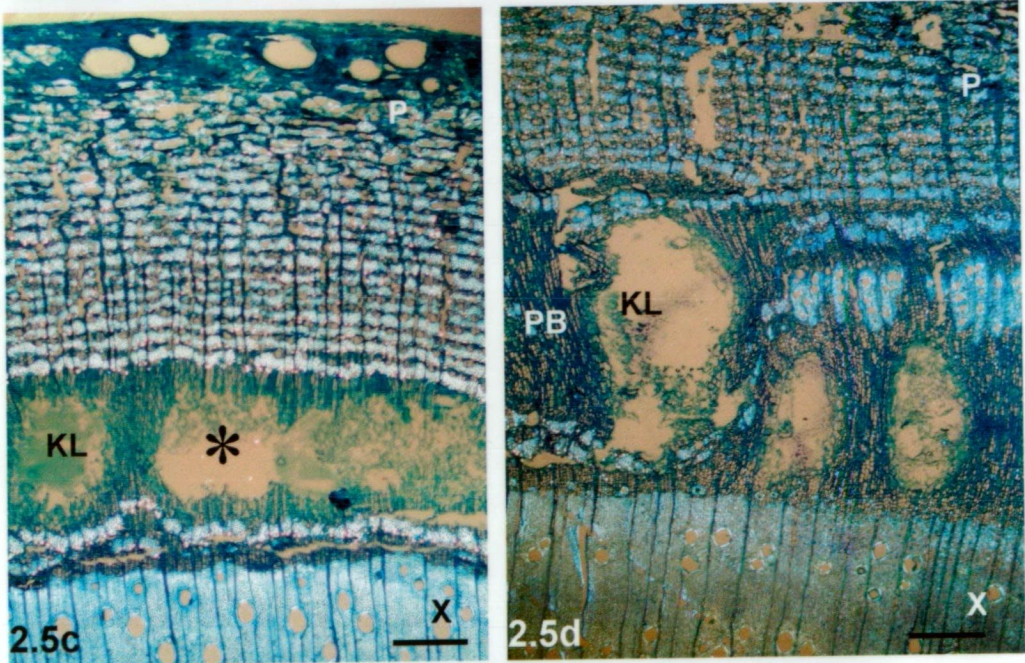
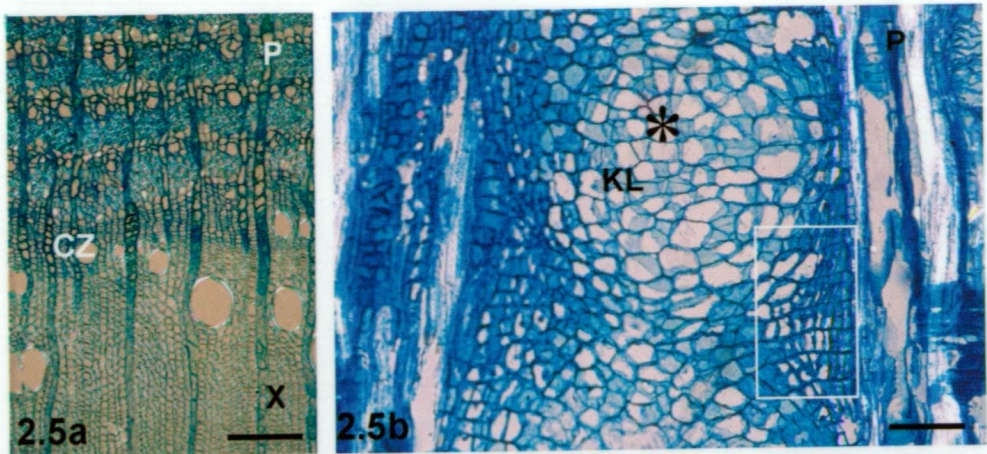
c: Disintegration of the central cells (*) within the kino lacunae observed 28 days post-induction. Bar = 500 μm .

d: Mature kino vein 35 days post-induction. The orientation of the vascular cambium (broken line) changes so that the kino vein appears to be included in both xylem and phloem. Bar = 500 μm .

e: Example of complex kino vein anatomy 35 days post-induction. Hemispherical protuberances formed on the outer edge of kino lacuna are located in the square. Bar = 500 μm .

CZ = cambial zone, PB = parenchyma bridge, KL = kino lacuna, P = phloem, PF = phloem fibres, X = xylem.

X



2.3.3 Response of *E. nitens* to CEPA treatment

Kino veins were not induced in juvenile *E. nitens* (Table 2.1) by CEPA treatment suggesting that this species does not readily form kino veins (Yang and Waugh, 1996). Even mature *E. nitens* trees treated with CEPA generally failed to produce kino veins (Eyles, personal observation). Instead, longitudinal sections of *E. nitens* 28 days after CEPA treatment revealed the production of around eight to ten tangential rows of a combination of apotracheal parenchyma and enlarged ray parenchyma (Appendix 2.2). This response was clearly different to that observed in the control. After 4 weeks, the wood was observed to be coloured a distinct pink. The pink coloured wood was treated with BBG-250. Fresh sections stained intensely blue indicating the presence of hydrolyzable tannins in this tissue (Fig. 2.6b). Resumption of normal wood production by the cambium occurred 35 days after CEPA treatment. The axial-like parenchyma and ray-like parenchyma tissue cells produced by juvenile *E. nitens* in response to CEPA treatment is very similar to the barrier zones formed in response to wounding and infection in other tree species such as beech (*Fagus sylvatica*) (Torelli and Oven, 1994). Here, abnormally large amounts of axial parenchyma characterized the barrier zone.

2.3.4 Kino vein development in *E. globulus* and *E. obliqua*

Approximately 7-14 days after CEPA treatment, the cambial initials had undergone rapid tangential and transverse division, producing broad bands of isodiametric thin-walled traumatic parenchyma cells. The central parenchyma cells accumulated polyphenols (Fig. 2.7a) and appeared to expand up to four times their original size. Subsequently these cells collapsed, most likely via a schizo-lysigenous process, to form the kino lacunae (Figs. 2.6c & 2.7b). Tissue described as 'parenchyma bridges' developed between the lacunae. The parenchyma bridge consisted of thin-walled radially elongated brick-like cells and the occasional crystalliferous parenchyma and phloem fibre-like cells (Figs. 2.6e & 2.7b). The parenchyma bridges tested negative for the presence of proanthocyanidins, indicating that these parenchyma bridges have no role in the synthesis of polyphenols (Fig. 2.6c). Instead,

the production of parenchyma bridges may serve to ensure the survival of the vascular cambium by forming a living link of cells between the xylem on either side of the tangential row of cavities, particularly since reversion to normal wood formation starts in the bridge tissue. During kino vein formation, the ray parenchyma remained intact if found within a wide parenchyma bridge. However, if the ray parenchyma was within the lacuna at the time of tissue differentiation, the ray cambial cells also produced traumatic parenchyma, similarly to that observed by Dowden and Foster (1973) in *E. radiata*. The two to four cell deep layer of parenchyma surrounding the lacuna became suberized approximately 5 weeks after treatment for *E. obliqua* and approximately 7 weeks for *E. globulus*. In comparison, Skene (1965) studying kino vein formation in mature *E. obliqua*, noted the production of suberized cells 7-8 weeks after wounding with a saw. The presence of suberized cells was successfully detected by Sudan black B dye and further confirmed by both fluorescence microscopy (Fig. 2.7c). The cambium resumes normal xylem formation following suberization of these cells.

In *E. globulus* trees only, the parenchyma cells at the outer edge of the kino lacuna underwent multiple divisions to produce concentric layers of epithelial cells that appeared to secrete kino into their vacuoles (Fig. 2.5e). Evidence of these specialized kino-secreting cells was provided by the DMACA test, which detected the presence of proanthocyanidins only in these cells (Figs. 2.6a and c). The innermost layer of cells lining the lacuna became greatly extended, subsequently crushing the thin walled cells located at the centre of the lacuna (Fig. 2.6a). The continuous division of the meristematic layer surrounding the lacuna then appeared to replace those compressed cells. The developing kino-forming cells were characterized by their large nuclei (Fig. 2.5b).

Fig. 2.6. Localization of phenolic compounds with Coomassie BBG-250 and DMACA stains.

a and c: Transverse section showing the concentric cells (arrows) lining the kino vein of *E.*

globulus staining positive with DMACA treatment, 35 days post-induction. No reaction

observed in the parenchyma bridge (*). Bar = 150 µm.

b: Transverse section showing localization of hydrolyzable tannins in the barrier zone of

E. nitens 49 days post induction with the Coomassie stain. Bar = 150 µm.

KL = kino lacuna, PB = parenchyma bridge, X = xylem.

Fig. 2.7. Transverse (a-c) and radial longitudinal (d) section showing kino vein development in *E. obliqua*.

a: Traumatic parenchyma produced by vascular cambium is observed to accumulate

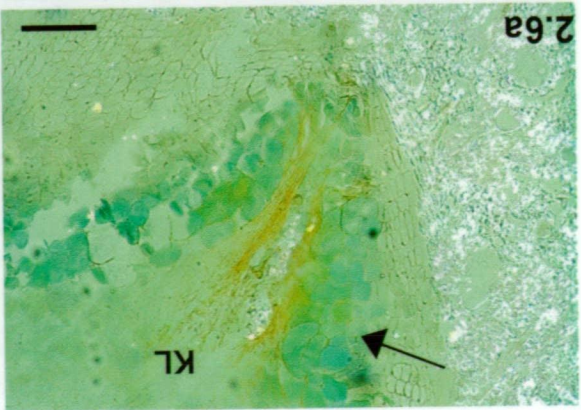
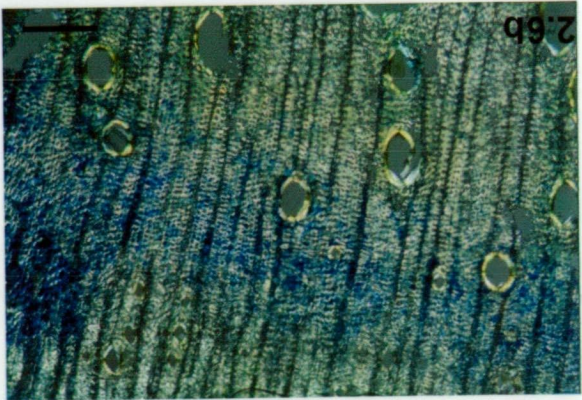
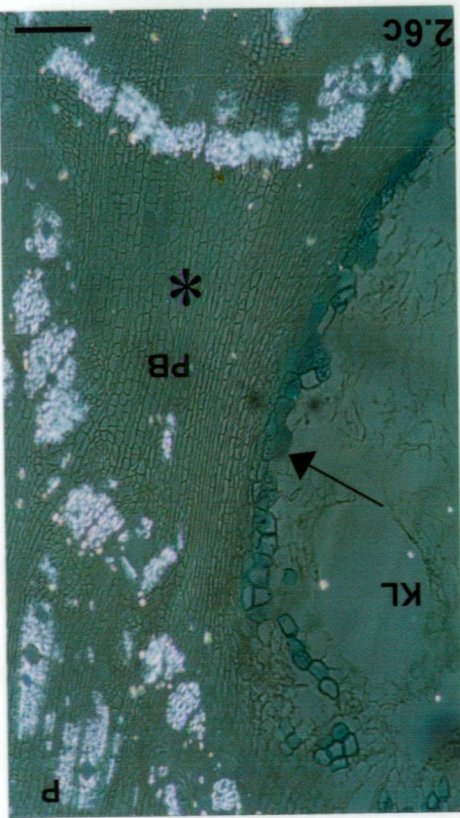
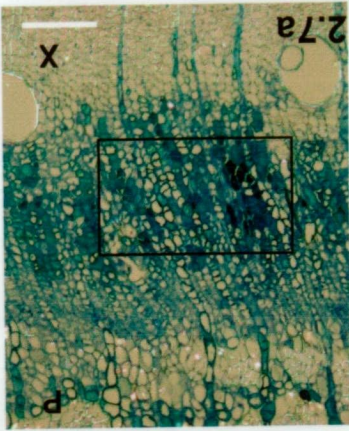
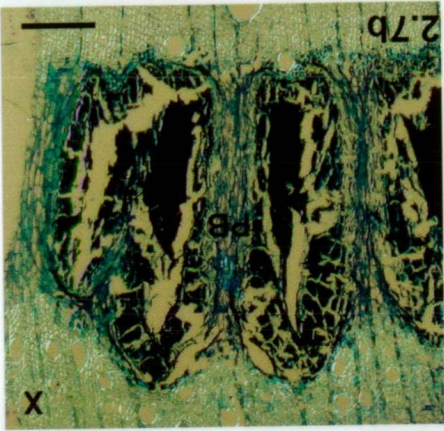
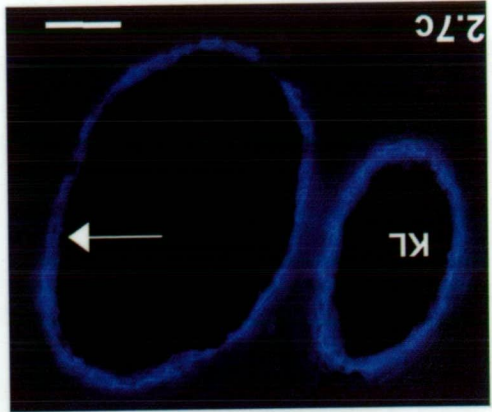
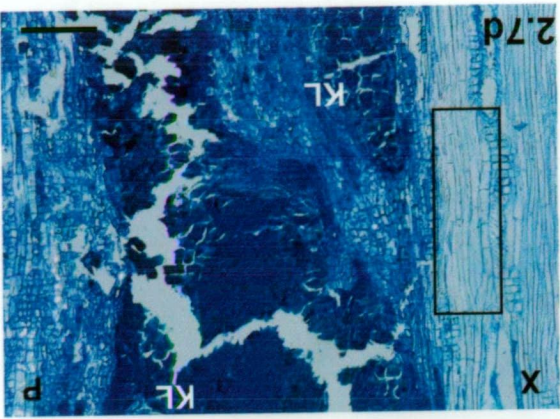
polyphenolic extractives (*) 14 days post-induction. Bar = 150 µm.

b: Mature kino vein in xylem 35 days post-induction. Bar = 200 µm.

c: Suberized cells (arrow) lining mature kino vein 42 days post-induction. Bar = 200 µm.

d: High number of axial parenchyma (square) along the pith side of kino vein observed 42 days post-induction. Bar = 150 µm.

CZ = cambial zone, KL = kino lacuna, PB = parenchyma bridge, P = phloem, X = xylem.



The ability of the parenchyma cells surrounding the lacuna to become meristematic and appear cambiform was likewise observed by Babu *et al.*, (1987) in their study of the development of traumatic gum-resin cavities in the xylem of *Ailanthus excelsa*. The workers suggest the adjacent meristematic cambiform cells contribute to the gum-resin formation by providing additional epithelial cells particularly during prolonged exudation. Based on our observations, the more complex description of kino vein formation as detailed above can also provide a possible mechanism to explain cases of prolonged kino production in the plant. Moreover, in some kino veins, the area of kino secreting tissues was increased by the formation of hemispherical or elongate protuberences on the outer edge of the lacuna (Fig. 2.5e). This latter anatomical feature was also observed by Dowden and Foster (1973).

The general structure of individual kino veins found in both *E. globulus* and *E. obliqua* appears to resemble those described in other eucalypt species, e.g. *E. camaldulensis* and *E. gomphocephala* (Day, 1959), *E. obliqua* (Skene, 1965), *E. radiata* (Dowden and Foster, 1973), *E. wandoo* and *E. marginata* (Tippett, 1986). However, one anatomical feature only briefly mentioned by Tippett *et al.*, (1983), but commonly observed in this study, was the presence of three to six tangential rows of axial parenchyma located on the pith side of the mature kino vein in both *E. globulus* and *E. obliqua* (Fig. 2.7d). As mentioned earlier in *E. nitens*, the increased production of axial parenchyma has often been characterized as part of the barrier zone wound response by many tree species (Tippett and Shigo, 1981; Shigo, 1984). Tippett and Shigo (1981) suggest that the living nature of barrier zones allow this specialized tissue to actively respond against injury and infection by a range of defence mechanisms such as production of polyphenols.

2.3.5 Origin of traumatic parenchyma

The origin of traumatic secretory structures induced in both the xylem and phloem of other tree species has been studied. For example, traumatic resin canals induced in the xylem of *Abies firma* are derived from xylem mother cells (Kuroda and Shimaji, 1984). This study showed that in *E. obliqua* trees, the traumatic parenchyma, which later develops into kino

veins and then becomes included in the xylem, similarly originates from the vascular cambium (Fig. 2.7a). In contrast, traumatic resin canals formed in the phloem of Japanese cypress (*Chamaecyparis obtusa*), are derived not from phloem mother cells but from a tangential band of parenchyma cells located in close proximity to the vascular cambium (Yamanaka, 1989). The derivation of traumatic parenchyma in *E. globulus* was similar to that observed *C. obtusa*, i.e. the kino veins that were induced in the phloem of juvenile *E. globulus* did not appear to originate from the vascular cambium but instead appeared to be derived from the meristematic activity of the phloem parenchyma (Fig. 2.5b and c). More specifically, kino vein development began with the radial proliferation of 20-30 cells of traumatic parenchyma in the first layer of the phloem parenchyma cells adjacent to the xylem while the vascular cambium remains intact, as a continuous layer (Fig. 2.5c). These results are in conflict with the findings reported by Tippet (1986) who studied the development of artificially induced kino veins in mature *E. marginata* and *E. wandoo*. Tippet concluded that the traumatic parenchyma originated from the vascular cambium, regardless of whether the kino veins became included in the phloem or xylem. The differentiation of 'phloem' fibres within the bands of the parenchyma was only briefly noted. In an attempt to clarify the role of phloem parenchyma in the production of kino veins induced in the phloem, future work may involve studying kino vein development in *Eucalyptus* species that induce kino veins in phloem only.

APPENDIX 2.1**Why is *E. nitens* different to other eucalypts?**

Findings from Chapter 2 confirmed previous observations that unlike other eucalypt species including *E. globulus* and *E. obliqua*; *E. nitens* does not readily form kino veins. We have proposed that the formation of kino veins plays a crucial role in the ongoing survival of the vascular cambium after damage. If we accept this hypothesis, then kino vein formation may be an adaptation to the relatively frequent fires experienced by eucalypts in the Australian environment. It is already widely recognized that eucalypts have developed a number of adaptations to fires. These include thick bark, the ability to produce epicormic bud shoots on the branches as well as the ability to form lignotubers that fully replace the dead tree after a severe fire event (Opie *et al.*, 1984). As outlined in chapter 1, *E. globulus* is predominantly a coastal species while *E. nitens* is a cool temperate species, suggesting that for the latter species, fire events may have been less frequent. *E. globulus* is considered fire tolerant as a consequence of forming lignotubers on seedlings and shooting from epicormic buds. In contrast, *E. nitens* with its thin bark and an inability to form lignotubers is generally considered to be intolerant of fires (Turnball and Pryor, 1984).

APPENDIX 2.2

Macrographs showing developmental anatomy of kino vein formation in *E. globulus* in response to CEPA treatment.

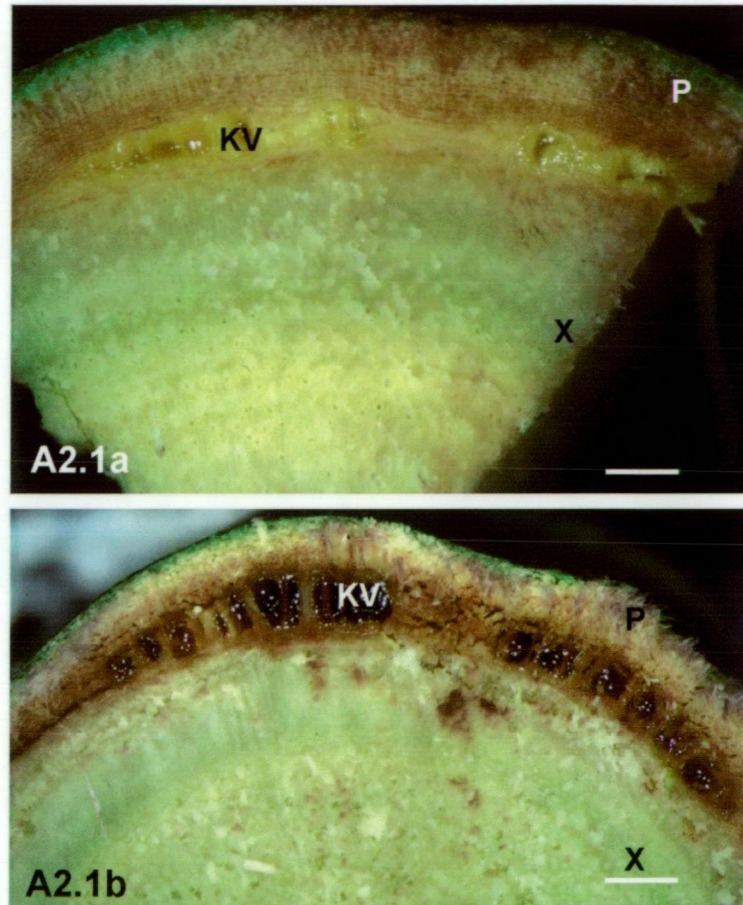


Fig. A.2.1. Transverse sections of kino vein development in the phloem of 3-year-old *E. globulus* treated with CEPA. a: 25 days post-induction with CEPA injection (note the straw colour of extractives) b: 35 days post-induction with CEPA injection (note the dark brown colour of extractives). All bars ~ 0.3 cm. KV = kino vein, P = phloem, X = xylem.

APPENDIX 2.3

Anatomical response of *E. nitens* to CEPA treatment.

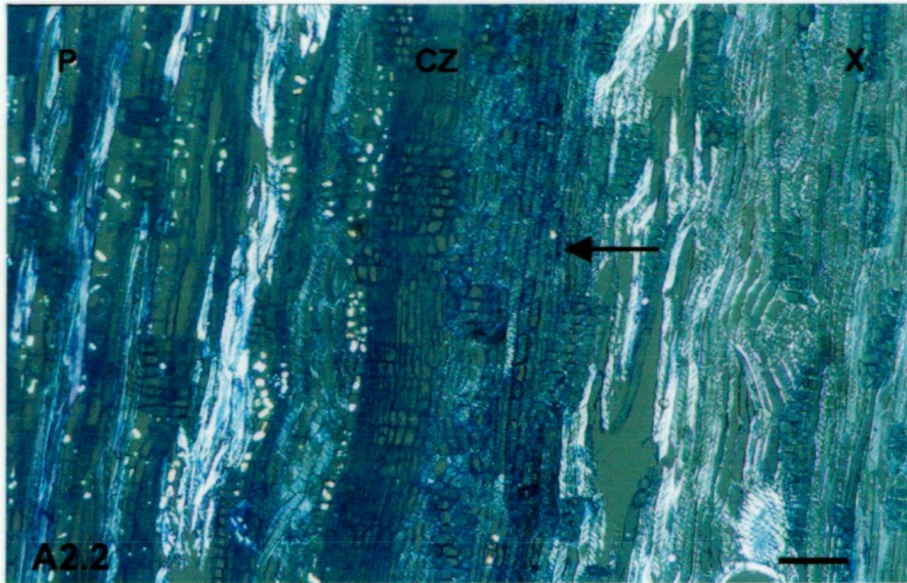


Fig. A2.2. Radial longitudinal section showing response of 2-3 year-old *E. nitens* to CEPA treatment, 21 days post-induction. Note anatomically different cells (arrow). Stained with toluidine blue. Bar = 100 μ m. CZ = cambial zone, P = phloem, X = xylem.

CHAPTER 3

Kino vein formation in *Eucalyptus globulus* and *E. nitens*

Abstract

Kino veins and/or pockets are characteristic defects of some *Eucalyptus* species and have long been recognised as sources of degrade in eucalypt timber. In order to investigate the causes of kino vein formation, the short-term responses of *Eucalyptus globulus* and *E. nitens* to mechanical, chemical (2-chloroethyl-phosphonic acid) and biological wounding treatments were examined. With the exception of the chemical treatment, the various wounding treatments did not consistently induce kino vein formation in either species. Instead, the new wound-associated wood and bark formed after wounding was characterized by the presence of dark extractives for both species. Although the dark extractives appeared macroscopically similar to that of kino, microscopic examinations showed the dark extractives to be formed in the less organized wound tissue and not in specialized kino veins. We suggest that the induction of tree exudates is part of a non-specific wound response and not necessarily induced as a direct host response to invasion by microbial pathogens and insects.

3.1 INTRODUCTION

The Tasmanian forest industry is increasingly applying intensive management practices to produce logs suitable for sawing from plantation eucalypts. Such practices include green pruning necessary for producing knot free wood quality as well as thinning to encourage rapid diameter growth in retained trees (Dudzinski *et al.*, 1992; Gerrand *et al.*, 1997).

However, one of the potential consequences of thinning and pruning operations is wounding to the retained trees, resulting in defect such as kino vein formation. Concern has been raised about the potential problem of sawlog downgrade due to a high incidence of kino veins (Gerrand *et al.*, 1997; Somervile and Davies-Colley, 1998; Vasiliauskas, 2001).

Kino veins and/or pockets are commercially regarded as characteristic defects of some *Eucalyptus* spp. (Bamber, 1985; Nelson and Hillis, 1978). Kino veins, more commonly known as 'gum veins,' can be the most severe forms of defect in the wood of some eucalypt species (Tippett, 1986). Somervile and Davies-Colley (1998) found that kino pockets had the potential to have a serious downgrading impact in veneer grade recovery in a stand of *E. regnans*. In addition, kino reduces yield, cause colouration problems and increase

chemical use in production of pulp (Hillis, 1984). Susceptibility to kino vein formation varies throughout the genus and with environmental conditions (Doran, 1975; Nelson and Hillis, 1978; Tippet, 1986). For example, trees growing on drier sites have apparently been observed to be the most severely affected by kino formation (Day, 1959). However, the exact cause of kino vein formation is still not clearly understood but it has been generally associated with a number of stresses that apparently damage but not kill the vascular cambium. These stresses include branch shed, damage by biological agents (e.g. insects and fungi), physiological stress, fire and mechanical damage (Jacobs, 1986). Most authors agree that kino veins generally play a role in wound defence as a tree resistance mechanism by acting as a 'barrier zone' (Wilkes, 1986; Hillis, 1987). Barrier zones have been defined as the production of specialised tissue by the cambium in a non-specific response to infection as well as to mechanical wounding and serve to compartmentalize necrotic sapwood from living cambium (Tippet and Shigo, 1981).

The present study aimed to conduct a descriptive study investigating the causes of kino vein formation in eucalypt tree defence by comparing the responses of two commercially important *Eucalyptus* species (*E. globulus* and *E. nitens*) to various mechanical, chemical and biological wounding treatments. Both *E. globulus* and *E. nitens* are widely planted in Tasmania for pulp and sawlog purposes (Beadle and Mohammed, 1999). Previous surveys of *E. nitens* sawlogs suggest that this species does not readily form kino veins (Nicholls and Pederick, 1979; Yang and Waugh, 1996a; Eyles and Mohammed, 2002) while a small amount of kino has been observed in sawn timber of *E. globulus* (Yang and Waugh, 1996b).

3.3 MATERIALS AND METHODS

3.3.1 Experimental site

Studies were conducted at two experimental plantation sites growing both *E. globulus* and *E. nitens*. The first site was located in Esperance (approximately 2 km south of Dover, Tasmania: lat. 43°15' S, long. 146°50' E). The altitude is 240 m a.s.l. The mean annual rainfall is 869 mm. The second site was in Lewisham (approximately 50 km east of Hobart,

Tasmania: lat. 42°49' S, long. 147°37' E). The altitude is 20 m a.s.l. The mean annual rainfall is 512 mm. Details of site establishment and tending for the Esperance and Lewisham sites are provided by Beadle *et al.*, (1996) and White *et al.*, (1998), respectively. At the Lewisham site, an additional study was also carried out to explore the effect of drought stress on tree wound responses. The site had been divided into 6 blocks (replicates), three designated as 'irrigated' and three designated as 'rain-fed'. Description of experimental design and irrigation treatment has been outlined elsewhere (Honeysett *et al.*, 1996; Worledge *et al.*, 1998). Briefly, the irrigated blocks received 1300-1500 mm/year while the rain-fed blocks received a total of 900-1100 mm/year including water supplemented by irrigation. Trees at Esperance were 16 years of age with an average stand diameter of 24 and 26 cm for *E. nitens* and *E. globulus*, respectively. The trees at Lewisham were 9 years of age. The average stand diameters of the irrigated trees were approximately 19 cm for *E. nitens* and *E. globulus* while the average stand diameters of the rain-fed trees were approximately 16 cm for *E. nitens* and *E. globulus*. In late summer of 2000, six trees each of *E. nitens* and *E. globulus* at Esperance and ten trees each of *E. nitens* and *E. globulus* (five from the irrigated block and five from the rainfed blocks at Lewisham) were randomly selected for wounding treatments.

3.3.2 Wounding treatments

The circumference of each tree was divided into 4 equal quadrants at approximately at 1.3 m. Four treatments were randomly assigned to the quadrants, one per quadrant as follows.

1. The impact force of a 3 kg steel rod (13 cm wide x 15cm long) attached to a 1 m long rope swinging through an arc of 90° (Fig. 3.1). This stimulated the 'bruising' type wound commonly inflicted on the retained trees during thinning operations.
2. 0.5 mL of 0.2 % CEPA aqueous solution (2-chloroethyl-phosphonic acid, SIGMA) was injected into a small wound (1cm²) created using a chisel. CEPA releases ethylene under physiological conditions. The application of exogenous ethylene to induce production of tree secretions has been well documented in many tree species including *Eucalyptus* (Dowden and Foster, 1973; Nelson and Hillis, 1978; Hillis, 1987). The anatomical and

histochemical responses to CEPA treatment were recently reported for juvenile

E. globulus and *E. nitens* (Chapter 2).

3. A 10 mm diameter hole was drilled 3cm into the outer sapwood in order to simulate the stem wounds commonly associated with stem boring insects. A second hole with identical dimensions to the first was drilled 30 cm above the first hole and inoculated with a decay fungus previously isolated from an *E. nitens* white-rot decay column. Inoculation involved inserting 2 x 1 cm² rectangular pieces of a 2-week-old culture growing on malt extract agar into the drill hole and sealing with a layer of lanolin wax. The decay fungus used for the inoculation was an unidentified basidiomycete known as 'Isolate D'. The fungus is described in Barry (2001).
4. A block of dry ice (approximately 7 cm²) was pressed firmly onto the bark for 5 min. This treatment attempted to damage the vascular cambium without physically disrupting the outer bark so as to minimize the exposure of injured tissue to the external environment (Robinson, 1997).

Approximately three months after treatment, 3 trees each of *E. nitens* and *E. globulus* at both the Esperance and Lewisham sites were harvested. Billets of approximately 50 cm in length that included the wounding treatments were taken to the laboratory. Each wounding treatment was excised from the billet by cutting longitudinally through the centre of the wounds using a bandsaw. The production of dark extractives was consistently observed as part of the wound response to varying degrees amongst all treatments. Therefore, responses were classified according to whether dark extractives (defined as non-structural components of wood and bark) were produced within specialized secretory cells i.e. kino veins or within new wound-associated wood formed directly adjacent to the wounding site (Fig. 3.3 and 3.4). For clarity, kino will refer to the exudate produced in kino veins while other exudates not formed in kino veins will be referred to as 'dark extractives'. In cases where kino veins had been formed, the length and width of the kino veins were recorded.

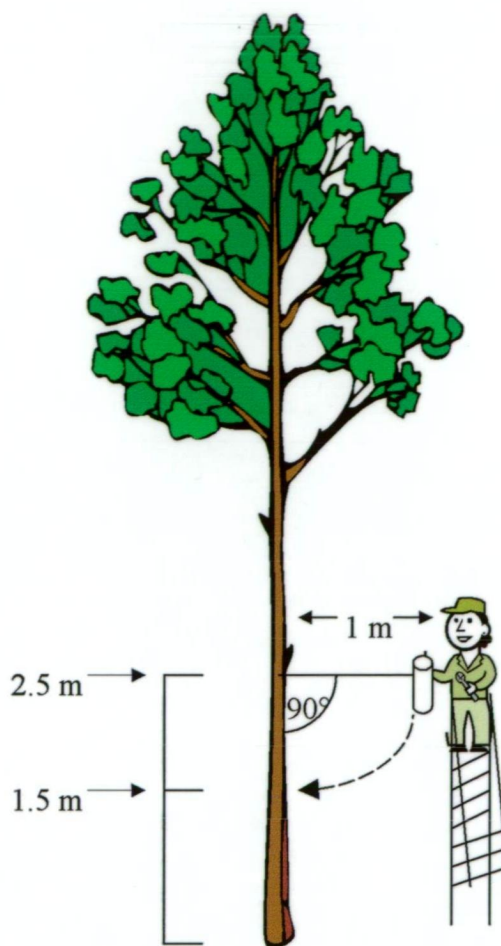


Fig. 3.1. Pictorial representation of the 'bruising treatment.' The 3kg steel rod attached to a rope tied to the stem was released from an angle perpendicular to the bole and allowed to freely swing down in an arc shape (dotted arrow) to hit the stem.

3.3.3 Light Microscopy

Wood samples of relevant wound response (c. 15 mm³) were fixed in FAA (formaldehyde:acetic acid:50% ethanol, 5:5:90 v/v/v) for a minimum of 24 hours at 4°C, dehydrated with an ethanol series and infiltrated in a L. R. White acrylic resin (Proscitech, Brisbane) series. Samples were then polymerized in fresh 100% LR White resin for 8-10 hours at 60°C (Chapter 2). Transverse and longitudinal sections of 10 µm in thickness were cut with a sledge microtome, dried onto glass slides, stained with a 1 % aqueous solution of toluidine blue and permanently mounted in Cytoseal (ProSciTech, Brisbane). Tissue structure and cell changes were noted on a Zeiss Axioskop photomicroscope. This procedure

was repeated with the remaining trees, approximately 13 and 17 months after setting up experiments at Esperance and Lewisham, respectively.

3.3.4 Data analysis

Since this qualitative study aimed to investigate the type of wounding treatments that may induce kino vein formation, the results were interpreted simply as positive or negative.

Therefore, the formation of kino veins regardless of size was regarded as a positive response to the wounding treatment. Only data from the CEPA treatment was considered for statistical analysis as kino veins were generally only formed in response to this chemical treatment. A

χ^2 contingency analysis was used to test whether kino vein incidence was species dependent.

Due to the low number of replicates, the data obtained from trees harvested at 3 months and those 17 months later at both the Esperance and Lewisham (including trees from the irrigated treatments only) were pooled.

3.4. RESULTS AND DISCUSSION

3.4.1 Response to CEPA treatment

The incidence of kino veins in response to CEPA treatment was significantly higher for *E. globulus* than *E. nitens* ($P < 0.001$). Kino veins were induced in the xylem of all eleven *E. globulus* treated with CEPA at both Lewisham and Esperance (Fig. 3.2a and Table 3.1).

The size of kino veins ranged between 15-177 cm in length and 4-7 cm in width.

Microscopic examinations showed that the general structure of kino veins produced in *E. globulus* was similar to those described for other eucalypt species, e.g. *E. obliqua* (Skene, 1965), *E. radiata* (Dowden and Foster, 1973), *E. wandoo* and *E. marginata* (Tippett, 1986).

Typical kino veins consisted of a tangential series of radial and transverse parenchyma bridges that inter-connected cavities filled with kino (Fig. 3.2b) (Appendix 3.1).

Kino veins were only induced in three out of the eleven *E. nitens* treated. Furthermore, in two of the three trees, the kino veins were less than 15 cm long and 4 cm wide. These results confirm findings from previous studies that unlike other *Eucalyptus* species studied

previously, kino veins are not readily induced in *E. nitens*. The CEPA treatment induced the production of a thin layer of dark extractives (ranging from 8-24 cm in length) within the phloem in half of the *E. nitens* replicates. Microscopic sections showed that this layer was anatomically distinct from kino veins. In contrast to the regular arrangement of kino veins, the thin layer of extractives was comprised of traumatic parenchyma of varying shape and size that stained strongly for the presence of polyphenolic compounds. Moreover, unlike kino veins, which are derived from the initials of the vascular cambium, the traumatic parenchyma of *E. nitens* were observed to originate from the de-differentiation of pre-existing phloem parenchyma.

Nelson and Hillis (1978) studying the involvement of ethylene in kino vein formation in *E. regnans*, found the area of kino veins produced was a positive logarithmic function of CEPA concentration. They recommended that 1 mL of 0.1% ethrel (a commercial preparation of CEPA) solution would be adequate for future studies in the production of kino veins. It could be argued that application of higher concentrations than those applied in the present study (0.5 mL of 0.2% CEPA) might have led to a higher incidence of kino veins, particularly in *E. nitens*. However, results from another study found that even injection of 0.3 mL of 1 % CEPA concentrations failed to induce kino vein formation in juvenile *E. nitens* trees (Chapter 2).

Table 3.1. Kino vein incidence by CEPA treatment for *E. globulus* and *E. nitens* (significantly different values (p<0.001) as denoted by different letters)

	Total number of kino veins induced by CEPA treatment	
SITE	<i>E. globulus</i>	<i>E. nitens</i>
Esperance	6/6	2/6
Lewisham	5/6	1/5
TOTAL	11/11 (a)	3/11 (b)

Fig. 3.2-3 Typical responses of different wound treatments (All radial longitudinal orientation).

3.2a: Production of kino veins (arrow) observed in the xylem 3 months after CEPA injection applied via chisel wound (*) in *E. globulus*.

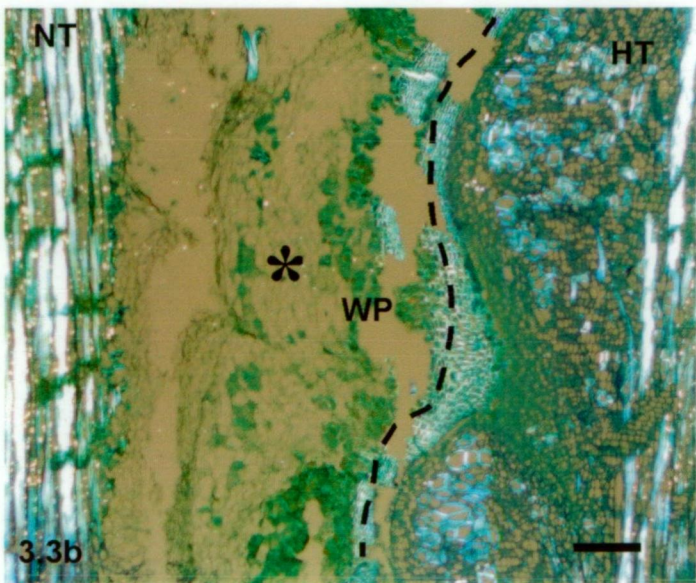
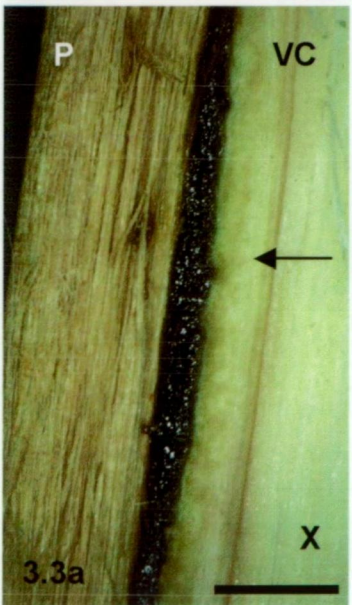
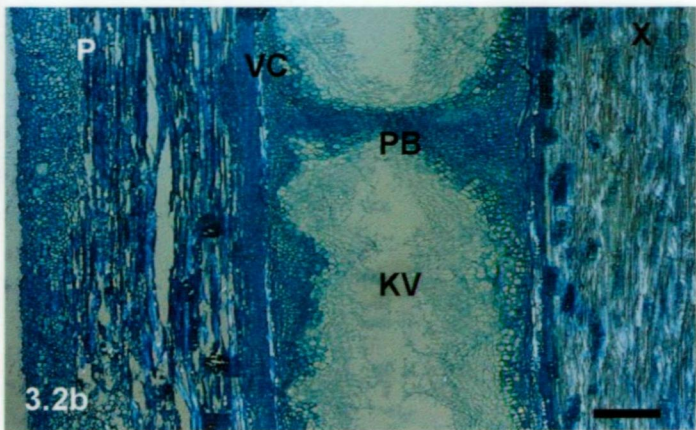
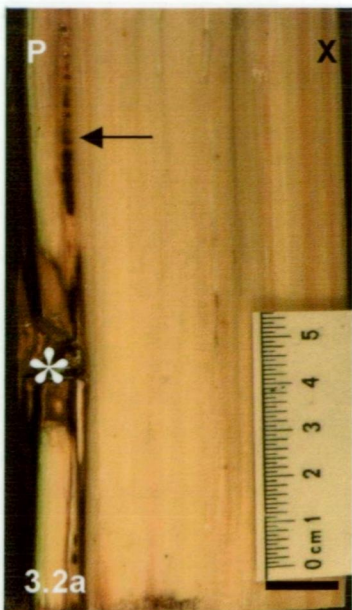
3.2b: Microtome sections of Fig 3.2a shown at higher magnification of regular anatomical arrangement of a typical kino vein.

3.3a: Production of a continuous layer of dark extractives (arrow) in the bark of *E. nitens* 3 months after dry ice treatment.

3.3b: Microtome section of Fig 3.3a shown at higher magnification of continuous wound periderm and disorganized formation of traumatic parenchyma (*) at interface between healthy and necrotic tissue.

Bar (3.2a and 3.3a) is ~ 1.5 - 2 cm. Bar (3.2b and 3.3b) is 500 μ m. PB = parenchyma bridge,

P = phloem, VC = vascular cambium, WP = wound periderm, X = xylem.



As mentioned earlier, in addition to observed genetic differences of kino vein susceptibility between species, environmental factors may also contribute to the incidence of kino vein formation (Hillis, 1987). Drier sites have been observed to be the most severely affected by kino vein formation and Day suggested that water deficiency during periods of active growth might explain this observation (Day, 1959). The effect of water stress on the incidence of kino vein formation has not been previously studied. In this exploratory study, kino veins were not induced in the xylem of any of the five *E. nitens* trees grown under simulated drought conditions. More significantly, kino veins were only induced in three out of the five *E. globulus* trees. While it is difficult to draw any firm conclusions from these results, they do appear contrary to Day's explanation, especially considering that water deficits inhibit division of fusiform initials and xylem and phloem mother cells (Kozlowski and Palladry, 1997). The relationship between water stress and kino vein formation warrants further investigations as future expansion of plantations may necessitate their establishment on water-limited sites.

3.4.2 Response to dry ice treatment

In a few cases, the dry ice treatment had destroyed the underlying vascular cambium while in the majority of other cases damage was restricted to within the phloem. The severity of damage did not appear to be directly related to bark thickness. Instead, differences in surface area contact and pressure applied in the dry ice treatments may explain the varying severity of damage even though every effort had been made to ensure even contact between dry ice and bark upon each application. The responses were generally characterized by the production of callus tissue with the deposition of a layer of dark extractives at the interface of healthy bark and bark that had been killed by the dry ice application. The amount of deposition was variable, appearing either as a continuous layer of variable width (Fig. 3.3a) or as irregular deposits along the necrotic margin. Microscopic examinations showed that the lesion margin was comprised of a completely differentiated wound periderm of multiple layers of thick-walled phellem in addition to traumatic parenchyma cells often filled with polyphenols, which had been derived from the de-differentiation of pre-existing phloem

parenchyma cells (Fig. 3.3b). Both *E. globulus* and *E. nitens* trees responded in this manner. However, some of the *E. nitens* trees at Lewisham produced different coloured extractives in both the xylem and phloem. Instead of the usual deposition of dark extractives, some *E. nitens* replicates produced a distinct pink coloured band of extractives located in the ray and axial parenchyma.

3.4.3 Response to drill wounding with or without fungi inoculation

Three months after treatment, both species generally responded to drill wounding with or without fungal inoculation by the production of callus tissue at the margins of the healthy tissue. The exposed callus was usually covered with thin film of dark extractives. At Lewisham, the drill holes of irrigated trees had been fully closed over by callus compared to those trees growing in the rainfed conditions. It would seem that the slower rates of wound closure as observed on the trees exposed to drought conditions may increase the duration of time when trees are exposed to microbial infection. Previous studies investigating the seasonal responses of *E. maculata* after artificial wounding found evidence to suggest that the rate of wound closure, whether by production of new wood or deposition of exudates, may be an important factor in restricting the colonization of wounds by decay fungi (Mireku and Wilkes, 1989). Furthermore, a clonal trial selecting for tolerance of *E. grandis* to a serious disease, *Cryphonectria* canker showed that clones with the fastest rate of wound closure were less susceptible to infection (Van Zyl and Wingfield, 1999).

Seventeen months after injury, all drill hole wounds (even those trees exposed to drought conditions at Lewisham) had fully occluded and the vascular cambium had been re-established. In both species, varying amounts of dark extractives were observed in the wound wood regardless of the whether fungal colonization had occurred or not (Fig. 3.4). Kino veins formed in response to drill wounding in four of the twenty-two *E. globulus* trees. Kino veins were not associated with drill wounding in any *E. nitens* trees. Drill holes inoculated with the unknown decay fungus had formed decay columns generally greater than 5 cm in length in both species. Fungal hyphae were visibly abundant in the drill hole and some were

observed to be in intimate contact with the inner side of the dark extractives (Appendix 3.2). Microscopic examination revealed that the hyphae had not spread into the new wound wood formed after wounding suggesting that the wound wood may be an effective barrier against further fungal colonization into the wood formed subsequent to damage.

3.4.4 Response to bruising treatment

In both species, the impact from the 3 kg steel rod generally killed the bark as well as the underlying vascular cambium that was located directly under the area of impact and surrounding margins. Subsequent to the impact, the overlying bark separated from the vascular cambium causing it to become detached from the bole while remaining on the stem (Fig. 3.5). Assessment of the level of damage was usually only possible after removal of the dead bark. In these cases, the bruising treatment was observed to cause extensive discolouration of the underlying xylem (Fig. 3.6). Furthermore, formation of kino veins (< 5 cm in length) and/or dark extractives were commonly observed along the outer edges of the callus margins, particularly in *E. globulus* trees. Another feature that was consistently associated with this treatment was the occurrence of bark splitting, which provided potential entry points for insect and fungi colonization (Fig. 3.5). Indeed, the presence of larvae and hyphae was observed for a number of trees in both species.

Despite the preliminary nature of this study, the results suggest that assessment of damage levels in the retained trees after thinning operations may be underestimated since bark covered wounds are not as visible as bark torn wounds. This finding may have important implications to industry, particularly as the development of decay and discolouration has been reported to occur at a greater rate in bark-covered than open wounds over a two-year study period (White and Kile, 1993; Dudzinski *et al.*, 1992). We suggest that any future work examining the contribution thinning operations may have on the incidence of kino vein formation should use a modification of the present bruising method. For example, the damage caused by a lighter steel rod may be more representative of the type of mechanical damage inflicted on the retained trees during thinning operations.

Fig. 3.4-7 Typical responses of different wound treatments.

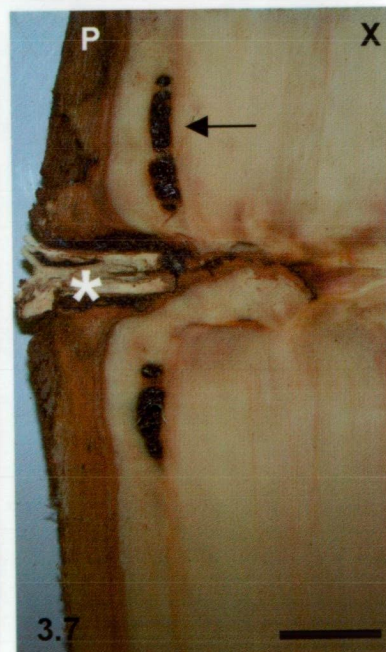
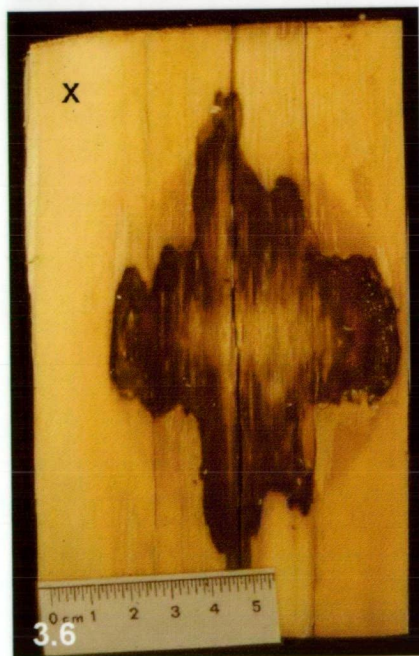
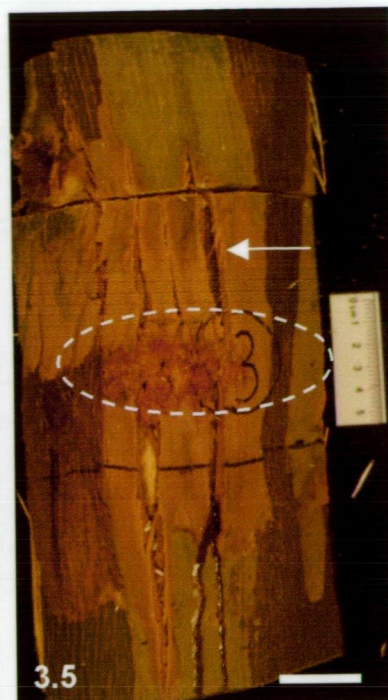
3.4: (Radial longitudinal orientation) Formation of wound wood and dark extractives (arrow)

17 months after drill wounding with decay (*) restricted to the sapwood present at time of wounding.

3.5 & 3.6: (Tangential orientation). The area of impact (circle) created by the bruising treatment resulted in the splitting of the bark (arrow) as observed in *E. nitens*. Removal of the bark revealed the discolouration of underlying xylem.

3.7: (Radial longitudinal orientation) Kino veins (arrow) induced by pruning wound in which the branch stub (*) was pushed out by subsequent growth in *E. globulus*. No sign of decay present. All bars ~1.5 - 2 cm.

P = phloem, VC = vascular cambium, X = xylem.



3.4.5 Role of kino and dark extractives in wound repair in eucalypts

In previous studies investigating eucalypt host-pathogen interactions, any exudate produced by the tree was simply referred to as kino (Mireku and Wilkes, 1989; Wardlaw, 1999). Kino was defined as ‘a red-brown aqueous polyphenolic exudate with the main constituent being polymerized forms of proanthocyanidins otherwise known as condensed tannins’ (Hillis and Carle, 1962; Hillis and Yazaki, 1975). Recent studies using modern analytical techniques including High Performance Liquid Chromatography coupled with Electrospray Ionization Mass Spectrometry (HPLC with ESI-MS) have shown the chemical composition of kino to be markedly different to that of dark extractives detected in wound wood (Chapters 4 and 5; Appendix 3.2). Given that there are clear differences in the chemistry and origin of these extractives, then we suggest that future studies investigating exudates in eucalypt defence should fully detail the nature of the exudates, particularly as these differences may reflect their varying roles in wound healing and defence. For example, in the present study, the production of dark extractives was shown to readily occur in either the xylem and/or phloem in both species, even in *E. nitens*, a species that does not usually form kino veins.

The production of tree exudates within specialized intercellular secretory spaces in the xylem and/or phloem is found in a range of woody tree species (e.g. latex from *Hevea* species, gum from *Prunus* species and resin from *Pinus* species (Hillis, 1987)). While exudates are generally implicated in protection against pests, diseases and damage, the precise nature of their involvement remains unclear (Pearce, 1996). There exists conflicting evidence of the role of kino vein formation as a defence mechanism in host-pathogen interactions. In one study, sizes of kino veins were significantly more extensive in trees inoculated with various canker fungi than those in control trees (Old *et al.*, 1986). Contrary to this report, kino vein formation was not inversely correlated with size of lesions formed in response to the root rot, *Phytophthora cinnamomi* in *E. marginata* (Tippett *et al.*, 1983; 1985). Given that fungal hyphae were observed to grow intimately with dark extractives (Appendix 3.3) as similarly seen by Wilkes (1986), it would seem that the induction of exudates in eucalypts is primarily in response to wounding and not specifically against biotic agents such as fungi. Even other

forms of wounding not considered in this experiment, such as pruning were shown to induce kino veins in the absence of fungal decay (Fig. 3.7). Hanks *et al.*, (1999) found no evidence to suggest that kino served to defend *E. rudis* trees against colonizing phloem-boring larvae (*Phoracantha semipunctata*), particularly as production of kino veins usually require days or even weeks whereas beetle larvae can penetrate the bark and reach the cambium within 24 hours. Studies investigating the induced wound reactions of Scots pine (*Pinus sylvestris* L.) to attacks by two bark beetles (Lieutier *et al.*, 1995) similarly concluded that the induced production of resin was solely a wound reaction induced by mechanical stress due to insect boring and not solely as a defence reaction against biological agents such as fungal pathogens. This does not mean however that these dark extractives may not have a possible role in impeding the spread of fungi or insects. For example, kino veins have been observed to be effective at preventing the outward spread of wood decay (White and Kile, 1993).

3.4 CONCLUSION

The production of exudates, either within the specialized kino veins or less organized wound-associated wood or bark was consistently observed as part of the host response to dry ice, bruising and drill wounding treatments, regardless of the presence of fungi colonization. For kino vein production, the integrity of the vascular cambium would appear to be the key factor determining their formation, as found in previous studies. Any stresses (whether mechanical, chemical or physiological) that damage but do not kill the vascular cambium may have the potential to produce kino veins in a susceptible species. Unlike other *Eucalyptus* species, kino veins are not readily induced in *E. nitens*, providing some evidence that this species is ideal for the commercial production of kino-free timber. In contrast, given that kino veins could be readily induced in *E. globulus* suggests, that for this species, kino vein defect may be a potential problem when grown for sawlogs.

APPENDIX 3.1

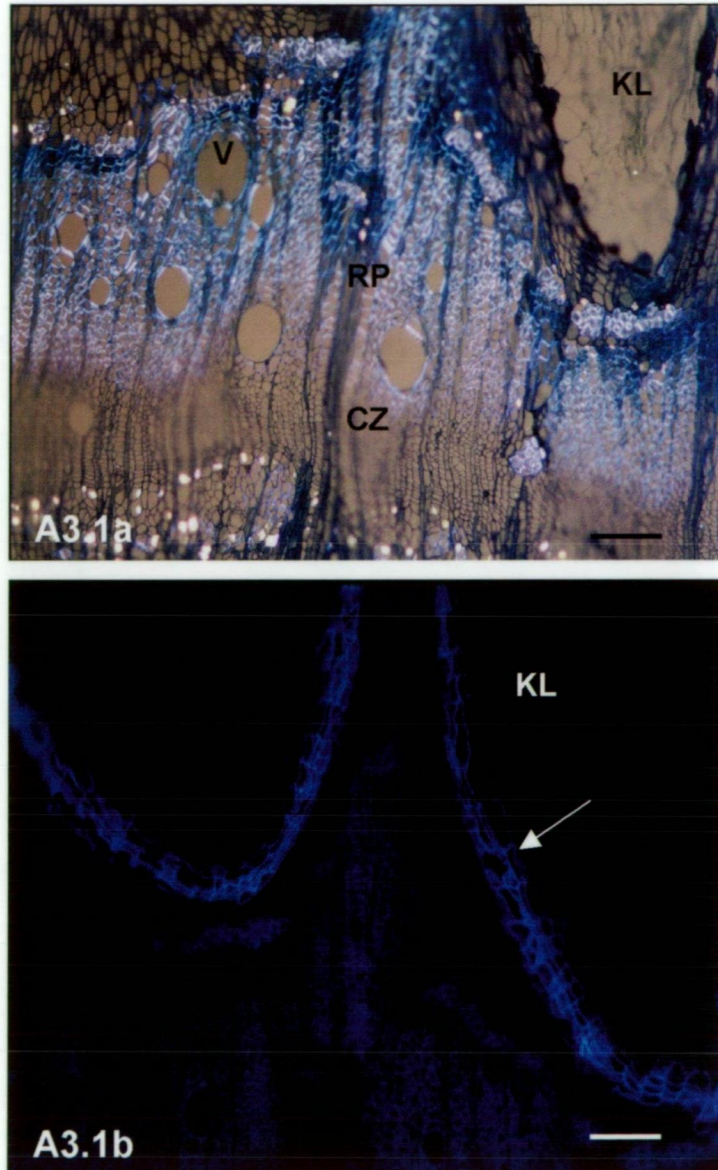
Anatomy of kino vein induced by CEPA treatment in 16-year-old *E. globulus*

Fig. A3.1a. Transverse section showing typical anatomy of a kino vein induced in 16-year-old *E. globulus* by CEPA treatment under light microscopy and under UV fluorescence (A3.1b). Note: kino lacuna is surrounded by 3-4 layers of blocky cells with suberized cell walls (arrow). Bar = 150 μ m. CZ = cambial zone, KL = kino lacuna, RP = ray parenchyma, V = vessel.

APPENDIX 3.2

HPLC Chromatogram of 70% aqueous acetone crude extract of kino exudate from *E. globulus*.

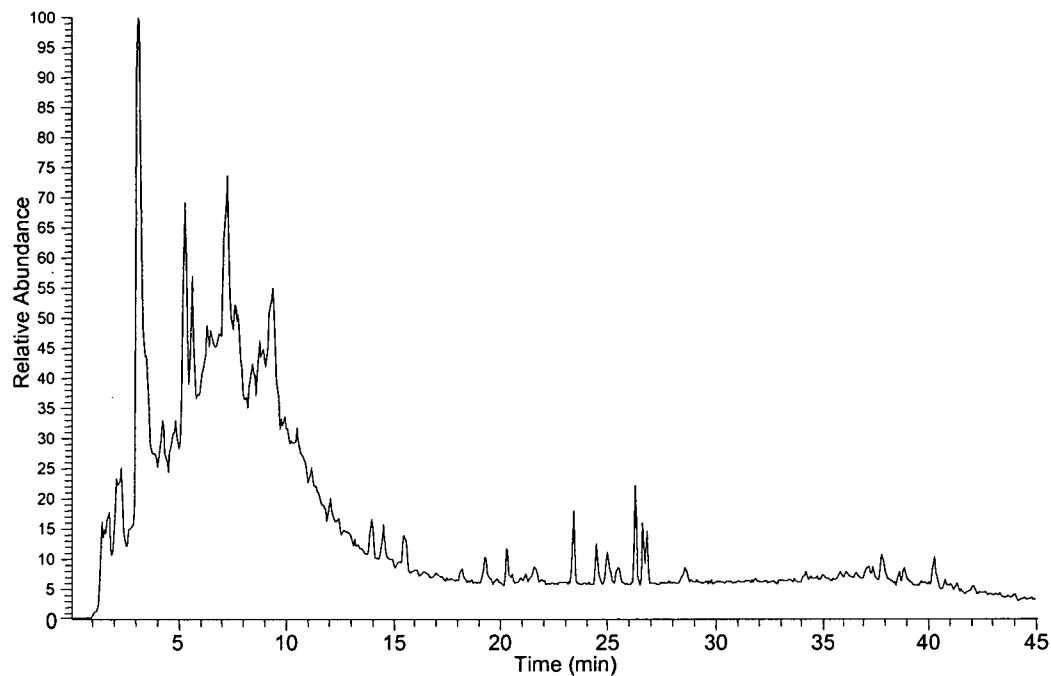


Fig. A3.2. HPLC chromatogram of 70% acetone *E. globulus* extracts of fresh kino detected at 280nm. In comparison to crude wound wood extracts (Chapter 4), fresh kino is not dominated by the presence of formylated phloroglucinol compounds (FPCs) as indicated by the lack of prominent peaks between 27-45 min.

APPENDIX 3.3

Fungal hyphae in intimate contact with dark extractives produced in response to drill wounding and fungal inoculation.



Fig. A3.3. Radial longitudinal section showing fungal hyphae (arrow) spreading over the inner side of the dark extractives. Bar = 3 mm. Extant sapwood is to the right of the figure.

CHAPTER 4

Wound wood formation in *Eucalyptus globulus* and *E. nitens*; anatomy and chemistry

Abstract

The wound-associated wood that developed 17 months following artificial xylem injury in *Eucalyptus globulus* and *E. nitens* was examined anatomically and chemically. This new tissue located immediately adjacent to the wound site and termed 'wound wood' was highly variable consisting of callus, altered wood of increased parenchyma density and dark extractives, visible to the naked eye. Subsequent chemical analysis of crude wound wood extracts by HPLC coupled to negative ion electrospray mass spectrometry revealed the presence of a diverse range of polyphenolic compounds including hydrolysable tannins, proanthocyanidins, flavonone glycosides and formylated phloroglucinol compounds. A number of polyphenols were unequivocally identified including engelitin, pedunculagin and tellimagrandin I. Other compounds present in wound wood include various hydroxystilbene glycosides and volatile terpenes. The importance of the diverse range of secondary metabolites detected in wound wood is discussed in relation to tree wound repair responses.

4.1 INTRODUCTION

Xylem wounding provides a major route for the entry of decay-causing fungi into living trees. Trees respond to wounding by 'compartmentalizing' the wounded area to limit the spread of dysfunction and infection (Shigo and Marx, 1977; Liese and Dujesiefken, 1990). Compartmentalization involves both biochemical and anatomical changes of the tissues present at the time of wounding and those that developed after wounding (Pearce, 1996, Schwarze *et al.*, 1999). Numerous investigators have studied the alterations in anatomy and histochemistry of xylem extant at the time of wounding and the new tissue formed by the vascular cambium known as the barrier zone (Bauch *et al.*, 1980; Rademacher *et al.*, 1984; Lowerts *et al.*, 1986, Rioux and Ouellete, 1991; Torelli *et al.*, 1994). In contrast, the new tissue formed immediately adjacent to the wound site (referred to as callus by Shigo, 1984) and directly involved in the closure of the wound has been given relatively minor consideration. We use the broader term 'wound wood' instead of 'callus' as the few studies that have examined the new tissue formed after wounding have found the wound wood to be

highly variable consisting of both callus and altered wood (Bauch *et al.*, 1980; Lowers *et al.*, 1986; Torelli *et al.*, 1994; Smith and Sutherland, 1999).

Successful and rapid wound closure may be an important factor in restricting the colonization of wound invading decay fungi. For example, in some tree species wound closure not only prevents further infections, but it is able to stop subsequent fungal development in already infected wounds, most likely by restricting gaseous exchange and moisture loss from compromised xylem thereby creating a microenvironment inimical to fungi colonization (Boddy and Rayner, 1983; Pearce, 1996). Despite its importance, the new wood formed after wounding has received little attention even in those northern hemisphere tree species that have been studied in more detail e.g. *Acer* (Bauch *et al.*, 1980) and *Juglans* (Smith, 1980). The few studies that have looked at the wood formed after wounding have focused on its histological characteristics and to date, no study has yet fully investigated its chemical nature. In particular, the chemistry of wound wood has never been previously studied in any *Eucalyptus* species. To date, more than two hundred non-volatile secondary metabolites have been reported from various species in this relatively large genus *Eucalyptus* L. Heritier (Myrtaceae) (>700 species) as reviewed by Singh *et al.*, (1999). The discovery of these compounds however, has generally been obtained from leaves, healthy wood and bark and kino, a polyphenolic exudate (Hillis and Yazaki, 1975; Cadahia *et al.*, 1997; Kim *et al.*, 2000; Close *et al.*, 2001). The only detailed study of the chemistry of sapwood in response to wounding and infection in eucalypts is that reported by Barry *et al.*, (2001 and 2002). Inoculation with decay fungi was shown to elicit increased levels of hydrolysable tannins.

The objectives of this study were to characterize, for the first time, the anatomical and chemical properties of the wound wood formed seventeen months following artificial injury in two economically important *Eucalyptus* species grown in southern Australia (*E. globulus* and *E. nitens*). Chemical analyses were performed by High Pressure Liquid Chromatography-Electrospray Ionization Mass Spectrometry (HPLC-ESI-MS), HPLC-UV and gas chromatography-mass spectrometry (GC-MS). LC-MS has proved to be a valuable

tool for the analysis of polyphenolics from crude and purified extracts (Nawaar *et al.*, 1997; de Pascual-Teresa *et al.*, 1998; Peuch *et al.*, 1999; Eschler *et al.*, 2000; Barry *et al.*, 2001; Davies and Barry, 2001). The complexity of the compounds detected in wound wood and their biological significance in relation to tree wound defence is discussed.

4.2 MATERIALS AND METHODS

4.2.1 Experimental site and wounding treatments

The experimental site growing both *E. globulus* and *E. nitens* (9-year-old) was in Lewisham, approximately 50 km east of Hobart, Tasmania (latitude 42°49' S, longitude, 147°37' E). The altitude was 20 m a.s.l.. White *et al.*, (1998) provide details of site establishment, fertilizer addition and pest control of the site. In the late summer of 2000, six trees each of *E. nitens* and *E. globulus* were randomly selected for wounding treatments. At breast height (*ca.* 1.5 m), a 10 mm diameter hole was drilled 3 cm into the outer sapwood. A second hole with identical dimensions to the first was drilled 30 cm above the first hole and inoculated with decay fungi previously isolated from an *E. nitens* white-rot decay column. Inoculation involved inserting 2 x 1 cm² rectangular pieces of a 2-week-old fungal culture growing on malt extract agar into the drill hole and sealing with a layer of lanolin wax. The decay fungus used for the inoculation was an unidentified basidiomycete known as 'Isolate D' and is described elsewhere (Barry, 2001). Briefly, this isolate has been associated with large decay columns resulting from pruning wounds and was shown to be a very aggressive pathogen in both pot and field pathogenicity trials.

Approximately seventeen months later, three trees each of *E. nitens* and *E. globulus* were harvested. Billets of *ca.* 50 cm in length that included the drill wounds were dissected with a bandsaw by cutting longitudinal sections through the wound. The responses to the drill treatments were noted. Chisel shavings (60-90 mg) of the wound wood, which only included new cells formed after wounding and had been produced adjacent to the drilling wound (Figs. 1a and 1b) as well as samples of healthy sapwood obtained from the same tree were prepared for LC-MS, LC-UV and GC-MS analysis. For LC-MS and LC-UV analyses,

wound wood samples were extracted twice with 750 µl of 70% aqueous acetone over 48 hours in the dark at 4 °C. The extract was then transferred to a 1.5 mL microcentrifuge tube and centrifuged (1300 rpm for 2 min) to remove solids. For GC-MS analysis, wound wood samples were extracted with 2 mL of dichloromethane over 24 hours in the dark at 4 °C. In both cases, samples were stored at –20°C and analyzed within one week of extraction.

4.2.2 Preparation of tissues for histological examination

Blocks (*ca.* 15 mm cube) obtained from the wound-associated wood located within the square shown in Fig. 4a were fixed in FAA (formaldehyde: acetic acid: 50% ethanol, 5 : 5: 90 v/v/v) for a minimum of 24 hours at 4°C, dehydrated with an ethanol series and infiltrated in L. R. White acrylic resin (Proscitech, Brisbane) series. Samples were then polymerized in fresh 100% LR White resin for 8-10 hours at 60°C (Chapter 2). Transverse and longitudinal sections of 10 µm in thickness were cut with a sledge microtome, dried onto glass slides, stained with a 1 % aqueous solution of toluidine blue and permanently mounted in Cytoseal (ProSciTech, Brisbane). Tissue structure and cell changes were noted on a Zeiss Axioskop photomicroscope. Suberin was detected with the autofluorescence quenching technique (Biggs, 1984). Autofluorescence of lignin was quenched with 1 % phloroglucinol-HCl and the residual distinct blue autofluorescence was observed under a Lietz fluorescence microscope equipped with a HBO 50-W mercury lamp with BP 340-380 excitation and LP 425 barrier filters. Monomeric flavan-3-ols as well as the corresponding oligomeric proanthocyanidins were detected by *p*-dimethylamino-cinnamaldehyde (DMACA) with a method modified from Gutmann (1993) described elsewhere (Chapter 2).

Fig. 4.1a and 4.1b. Macrograph (radial longitudinal) illustrating typical response to drill and inoculation treatment after 17 months in 9-year-old-*E. globulus* and *E. nitens*. Outside bark in all figures is orientated towards the top of plate while dashed red line indicates boundary between extant wood and new wound wood formed after wounding. Bar ~ 1 cm.

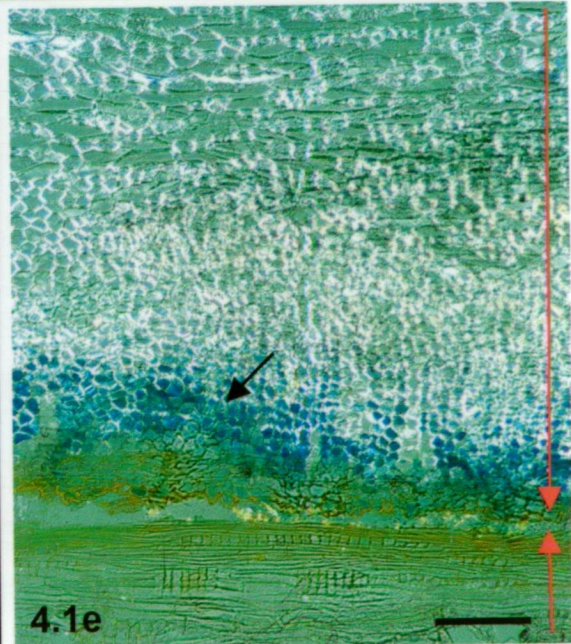
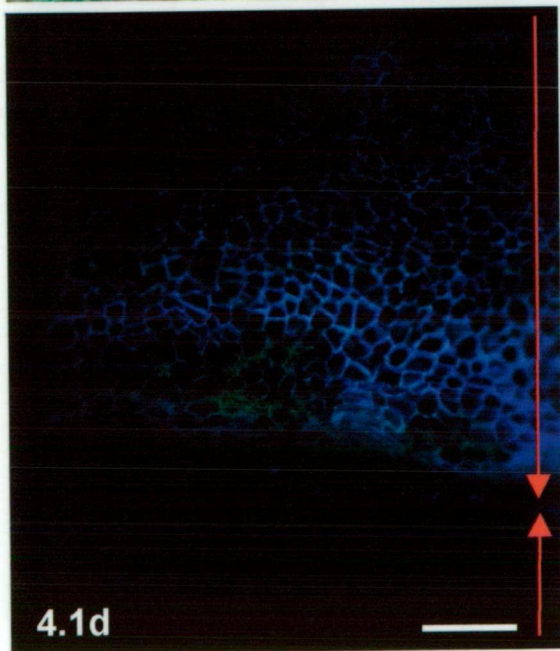
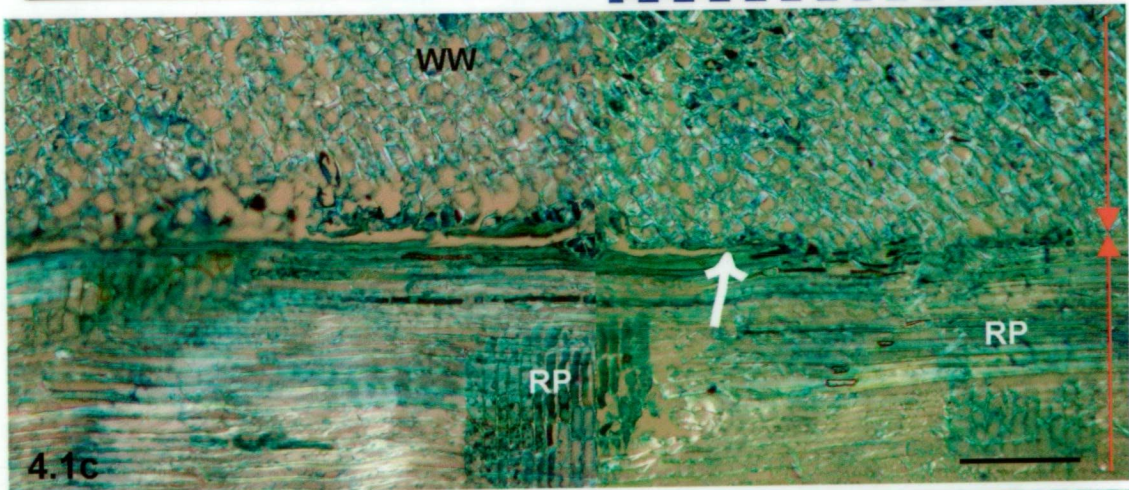
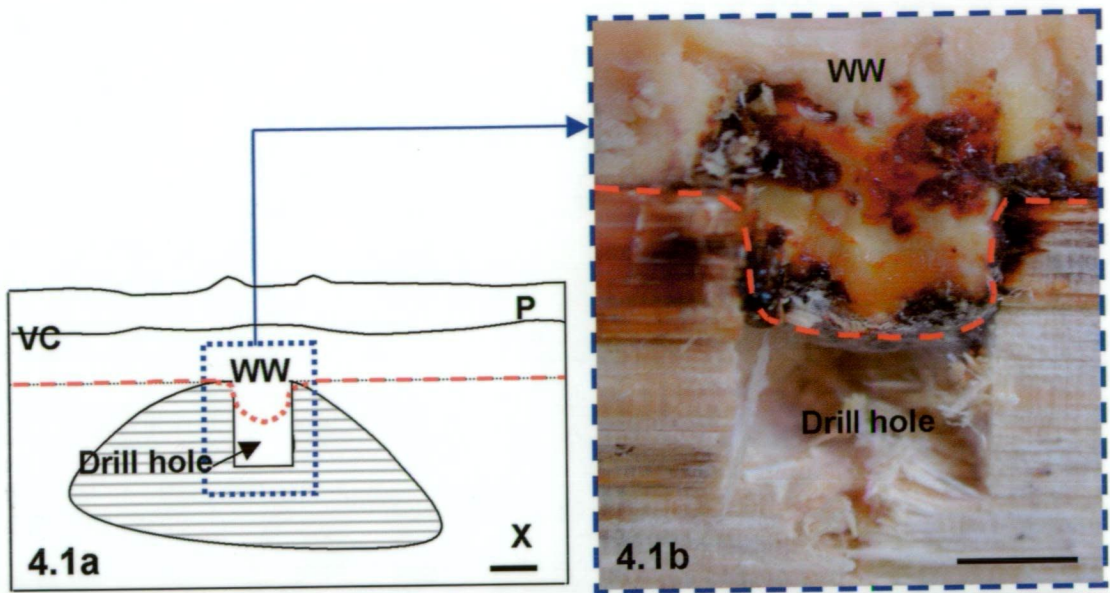
4.1c. Light micrograph of wound wood produced from the proliferation of healthy parenchyma at the tip of cambial dieback (white arrow) (stain: toluidine blue).

4.1d. Autofluorescence of wound wood stained with phloroglucinol-HCl under UV-excitation (410-nm barrier filter), indicating presence of suberin in cell walls.

4.1e. Similar section stained with DMACA indicating presence of proanthocyanidins (arrow).

Bar in 4.1c -4.1e = 200 μ m.

Note. Descending red arrow indicates new wound wood cells while ascending red arrow indicates extant sapwood. P = phloem, RP = ray parenchyma, VC = vascular cambium, WW = wound wood, X = xylem.



4.2.3 Chromatographic and spectrometric analysis

HPLC-UV: Injections of 5 μL of sample were analysed by HPLC with ultraviolet detection (HPLC-UV) with the following system. HPLC analyses were carried out on a Waters Alliance 2690 with a Waters 996 Photodiode Array Detector (Waters Corporation, Milford, MA, USA). The column was a Waters Nova-Pak C18 column (150 mm x 3.9 mm) fitted with an Alltech Econosphere 5 μm C-18 guard cartridge (Alltech, Melbourne, Australia). The flow rate was 0.8 mL min^{-1} . The solvent system was: solvent A, 2% acetic acid in methanol; solvent B, 2% acetic acid in distilled water; solvent C, 100% hexane. The solvent gradient was as follows: 10% solvent A/90% solvent B; ramped to 85% solvent A/15% solvent B at 25 min; then to 100% solvent A at 35 min; then to 80% solvent A/20% solvent C at 45 min. The original conditions were re-established by ramping to 100% solvent A for 2 min followed by 15-min re-equilibration at initial conditions. The photodiode array detector was monitored from 240 to 445 nm at a sampling rate of 1 spectrum s^{-1} and a resolution of 2.4 nm. The HPLC sample chamber was maintained at 5 $^{\circ}\text{C}$ throughout the analyses.

HPLC-MS: The HPLC column and conditions were described as above. The HPLC column outlet was coupled directly to a Finnigan LCQ ion trap mass spectrometer fitted with an electrospray source (Finnigan MAT, ThermoQuest, Co., San Jose, CA, USA). The instrument was operated in the negative ion mode, scanning from m/z 125-1500 with an automatic gain control target value of 2×10^7 and maximum ion injection time of 100 ms. Operating conditions were as follows: sheath gas 90 psi, aux gas 50 psi, ESI needle voltage 4.5 kV, capillary temperature 270 $^{\circ}\text{C}$ and capillary voltage -30 V. Data- dependent MS/MS spectra were routinely acquired from the most intense ion in the spectrum, with collision energy of 30% and a peak isolation width of 4 amu.

GC-MS: Analysis of the dichloromethane extracts was performed by combined GC-MS using HP-5890 GC directly coupled to a Hewlett Packard 5970 B Mass Selective Detector. The fused silica GC column was a 25 x 0.3 mm diameter HP1 (0.17 μm film) and the oven

temperature held at 20 °C for 2 min and then programmed to 240 °C at 6 °C min⁻¹, using helium as the carrier gas. Identification of the individual compounds was based on an 'in-house' library of reference mass spectra built up from standard compounds and oils of known composition and published Kovat's retention indices (Davies, 1990). Splitless injections of 1 µL were made at an injection temperature of 220 °C.

4.2.4 Depolymerization of the crude wound wood extracts in the presence of phloroglucinol

In order to determine the identity of unknown compounds that eluted from the HPLC-UV as a broad unresolved peak (Fig. 4.2), the crude extracts were acid hydrolysed (Pascual-Teresa *et al.*, 1998). For 2 samples each from *E. globulus* and *E. nitens*, 100 µL of original wound tissue crude extract was placed in a glass Reactivial (Alltech Associates Australia Pty Ltd.) together with 300 µL of phloroglucinol reagent (80 mg phloroglucinol dissolved in 0.5 mL methanol and 1.5 mL 0.5 M HCl). The mixture was allowed to react for 5 min in a water bath at 100°C. It was then cooled in water and later placed in a freezer to precipitate excess phloroglucinol. The depolymerized samples were analysed by LC-MS using the method described above.

4.2.4 Identification and semi-quantification

The identification of compound classes and specific compounds within them was achieved by a combination of interpretation of MS and tandem MS data from first principles (Stobiecki, 2000; De Freitas *et al.*, 1998; Hammerstone *et al.*, 1998), UV spectra, and comparison of HPLC, UV and MS data with standards where possible. The various hydrolysable tannins were identified by comparing their negative ion ESI-MS characteristics with those previously described by Barry *et al.*, (2001) and Davies and Barry (2001), who included the use of several authentic standards.

Calculations of the relative abundance of compound classes were based on the peak area of the HPLC-UV chromatogram, with some assignment in regions of co-elution, referred back to individual MS responses. An arbitrary classification system based on the approximate percentage of the total absorbance at 280 nm was then used to allow comparisons between wound wood and healthy sapwood (see Table 4.1).

Authentic standards

Purified standards of engelitin and resveratrol glycoside were used in the study. Standards were weighed and dissolved in 70% acetone at concentrations of 0.10 mg/100 μ L.

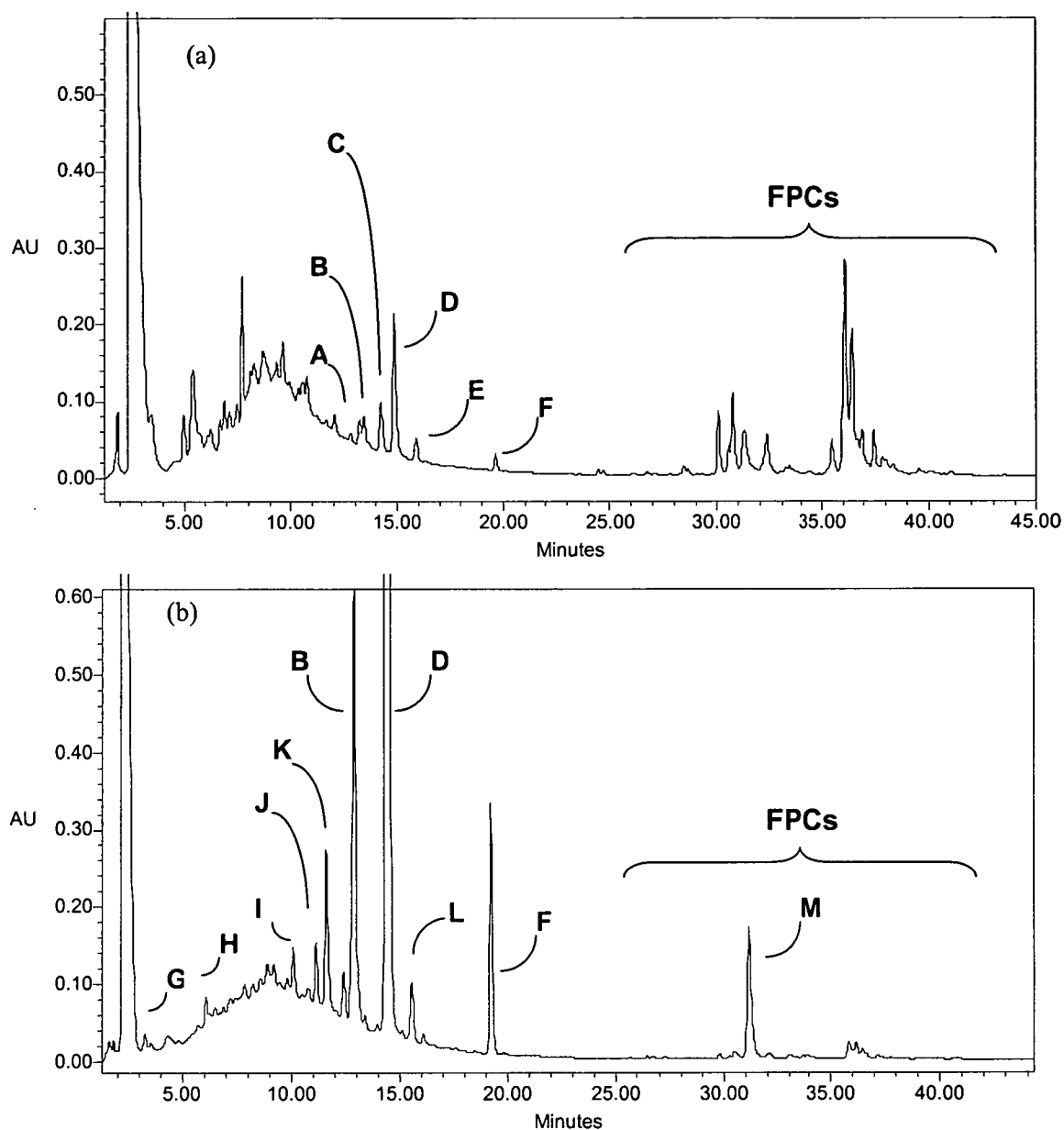


Fig. 4.2 Examples of typical HPLC-UV chromatograms of wound wood extract (70% acetone) of *Eucalyptus globulus* (a) and *E. nitens* (b) measured at 280 nm indicating major and minor compounds. The unresolved peaks between 5 and 20 min, largely contain (proanthocyanidins) PAs and hydrolyzable tannins. The peaks between 25- 45 min (within bracket) largely contains formylated phloroglucinol compounds (FPCs). Identities of peaks are as follows A. unknown flavonoid B. dihydroquercetin rhamnoside C. dihydroquercetin arabinoside D. engelitin, E. unknown flavonoid, F. dihydroquercetin, G. pedunculagin, H. catechin, I. tetra-galloyl-glucose, J. unknown stilbene glycoside, K. unknown stilbene glycoside, B. dihydroquercetin rhamnoside, D. engelitin, F. unknown flavonoid, L. unknown compound, M. sideroxylonal.

4.3 RESULTS

4.3.1 Morphological observations

In the 17 months following injury, all drill hole wounds had fully closed over and re-establishment of the vascular cambium was achieved (Fig. 4.1a). Moreover, all drill holes inoculated with the unknown decay fungus were shown to form decay columns (>10 cm in length) in *E. globulus* and *E. nitens*. The new tissue formed directly adjacent to the drill site termed 'wound wood' was usually characterized by the presence of dark extractives (non-structural components of wood), regardless of whether the inoculated fungus had caused decay or not (Fig. 4.1b). In all cases, no decay symptoms were evident in the wound wood. Moreover, fungal hyphae were not observed in the wound wood under light microscopy.

From histological studies, the wound wood was found to be highly variable comprising of callus production proximal to wounds while distal to the wound, the formation of 'wood,' was characterized by altered cell orientation, reduced vessel numbers and increased parenchyma density. The wound wood was observed to originate from the callus-like proliferation of healthy ray and axial parenchyma cells adjacent to the wounding site (Fig. 4.1c). The cell walls of the wound wood located directly adjacent to the dead extant xylem exhibited a distinct blue-white fluorescence indicating the presence of suberin (Fig. 4.1d). Furthermore, in contrast to extant sapwood, the innermost 10-15 layers of wound-associated wood located along the extant xylem stained strongly positive with the DMACA treatment, suggesting the presence of proanthocyanidins (Fig. 4.1e). In light of these results, the wound wood was analyzed by HPLC with ESI-MS in an attempt to elucidate the chemistry of the wood extractives.

4.3.2 Chemical response

Analysis of *E. nitens* and *E. globulus* crude wound wood extracts by GC-MS, HPLC-UV and HPLC-MS using negative ion electrospray ionisation indicated the presence of a very complex range of organic compounds which were either undetectable in healthy tissue or present at only trace levels. These included flavonoids and flavonoid glycosides, condensed

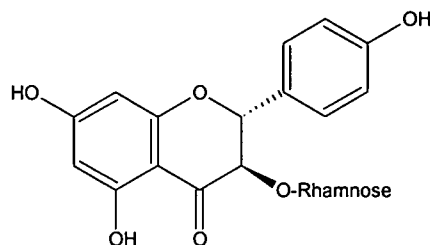
tannins, hydrolysable tannins, formylated phloroglucinol compounds (FPCs) (Chapter 5), terpenes and hydroxystilbene glycosides (Fig. 4.2, Table 4.1). Qualitative comparisons of crude extracts showed that the same diverse range of secondary metabolites was detected in wound wood obtained from trees that had been inoculated with decay fungus to those without inoculation.

4.3.3 Compound identification

Proanthocyanidins in wound wood: The wound wood crude extracts of both species appeared to be predominantly composed of unidentified compounds of high molecular weights observed as a broad unresolved region in the HPLC-UV chromatogram (Fig. 4.2). The bulk of these unknown compounds were provisionally identified as belonging to the major group of natural polyphenolic compounds known as proanthocyanidins (PAs; *syn*: condensed tannins). From interpretations of MS behaviour, the identities of a number of compounds were confirmed including catechin, procyanidin dimers, trimers and their respective gallates (Table 4.1). Preliminary analysis of other unknown PAs by acid-catalyzed cleavage with phloroglucinol as the nucleophile, resulted in a complex range of products that did not correspond to simple catechin/epicatechin (C/E) monomers or dimers bound to phloroglucinol. Direct interpretation of MS and tandem MS data indicated that the products observed could be readily explained, however, by almost complete substitution along the oligomer/polymer backbone with rhamnose and gallic acid.

Flavonone glycosides in wound wood: The principle flavonoid glycoside detected in wound wood crude extracts (particularly in *E. nitens* samples) was identified as engelitin (dihydrokaempferol 3-rhamnoside, Structure I) (Fig 4.2, Table 4.1). Its identity was established initially on the basis of molecular weight then tandem mass spectral data which indicated a flavonoid aglycone of apparent molecular weight 288 bound to rhamnose, and finally, unequivocally by direct comparison with an authentic standard. Other related flavonoid glycosides present in notably lower amounts included dihydrokaempferol arabinoside, dihydroquercetin glucoside and dihydroquercetin rhamnoside. These were

identified on the basis of UV, MS and tandem MS evidence only and not by comparison with authentic standards. For example, in the negative electrospray tandem MS data, rhamnose substitution was indicated by losses 146 and/or 164 daltons while arabinose substitution was indicated by losses of 132 and/or 150.



(I) Engelitin

Comparison of the relative abundance of compounds between species and tissue type:

Representative HPLC chromatograms of the crude wound wood extracts of *E. globulus* and *E. nitens* showed the crude extracts to be largely composed of PAs in addition to at least five other compound classes (Fig. 4.2). Of these other compound classes, *E. globulus* crude wound wood extracts contained higher levels of terpenes and FPCs than those from *E. nitens* wound wood. Conversely, crude extracts from *E. nitens* wound wood contained higher levels of the flavanone glycosides (particularly engelitin) and hydrolysable tannins than found in the crude wound wood extracts of *E. globulus*. In contrast, with the exception of a dihydroquercetin arabinoside and hydrolysable tannins detected in only trace amounts, the other compound classes were not detected in healthy sapwood (Table 4.1).

Table 4.1. Relative abundance of the representative compounds present in crude wound wood extracts of *Eucalyptus globulus* and *E. nitens* as determined by HPLC-UV and supported by HPLC-MSⁿ data.

COMPOUND CLASS	[M-H]	HS	Relative abundance ^A Species		
			<i>E. globulus</i>		<i>E. nitens</i>
			WW	HS	WW
Flavanone					
dihydrokaempferol*	287	0	0	0	+
quercetin*	301	0	tr.	0	+
Flavanone glycosides					
engelitin*	433	0	+	0	+++
dihydroquercetin arabinoside	433	tr.	tr.	tr.	tr.
dihydrokaempferol arabinoside	419	0	0	0	+
dihydroquercetin rhamnoside	449	0	0	0	+
Proanthocyanidins					
catechin*	289	0	+	0	+
catechin gallate	441	}	+	0	+
procyanidin dimers	577				
unknown proanthocyanidin	723				
procyanidin dimer gallates	729				
procyanidin trimer gallates	1017				
unknown proanthocyanidin	1125	}			
unknown proanthocynidin	1157				
polymeric proanthocyanidins	>1200	0	+++++	0	++++
Hydrolyzable tannins					
methyl ellagic acid arabinoside	447	0	+	0	+
trigalloyl glucoses	635	0	+	tr.	+
pedunculagin	783	tr.	+	tr.	++
tellimagrandin I*	785	0	tr.	0	+
tetragalloylglucoses*	787	0	tr.	tr.	++
unknown ellagitannin	933	0	+	0	+
pentagalloylglucose*	939	0	+	tr.	+
Formylated phloroglucinol Compounds (FPCs) ^B					
		0	+++	0	++
Stilbene glycosides					
resveratrol glycoside	389	0	0	0	+
astringenin	405	0	tr.	0	tr.
a trihydroxy-methoxystilbene glycoside	419	0	0	0	+
Terpenes ^C					
1,8-cineole *		0	###	0	####
α-pinene *		0	##	0	##
globulol *		0	##	0	#
aromadendrene *		0	##	0	#

^A Relative scale where 0 = none detected; Tr. (trace amount) = 0-1; + = 2-5; ++ = 6-15; +++ = 16-25; ++++ = 26-50, +++++ = >50. ^B See Eyles *et al.*, 2002. ^C Relative abundance of terpenes as determined by GC-MS (based on the same relative scale used for the other compound classes but noted by #). * Identity confirmed with standard. HS = Healthy sapwood, WW = wound wood.

4.4 DISCUSSION

Histological examinations showed the wound wood of both *E. globulus* and *E. nitens* to be highly variable displaying similar morphology to that reported for other tree species i.e. comprising both callus and altered wood (Bauch *et al.*, 1980; Lowerts *et al.*, 1986; Torelli *et al.*, 1994). In addition, the wound wood was also characterized by the presence of visually obvious dark extractives. From gross morphological comparisons of the wound wood observed in the two eucalypt species with that reported for other woody angiosperms, the production of such obvious extractives appears to be a unique feature of eucalypts. For example, no such production of extractives was reported to be present in the new tissue formed after drill wounding in black walnut (*Juglans nigra* L.) (Smith, 1980) nor in other woody tree species (Bauch *et al.*, 1980; Lowerts *et al.*, 1986; Torelli *et al.*, 1994; Smith and Sutherland, 1999).

Subsequent chemical analysis of the crude extracts obtained from the wound wood revealed the presence of a complex array of secondary metabolites. More specifically at least six classes of secondary compounds were detected in the crude wound wood extracts of both *E. globulus* and *E. nitens* compared to the trace levels of two compound classes detected in healthy sapwood. Interestingly, most of the PAs detected in wound wood were not of the usual catechin/epicatechin (C/E) monomers or oligomers but were more complex PAs, substituted with rhamnose and gallic acid. The presence of 3-O- α -L-rhamnopyranosyl- (+)-catechin monomer units in condensed tannins has been previously reported in mangrove bark (Achmadi *et al.*, 1994). These were also detected as their (4 α \rightarrow 2) phloroglucinol adducts from a depolymerisation reaction. Furthermore, rhamnosides and gallates are well known in simple flavanol monomers and dimers (Harborne and Baxter, 1999).

The findings from the present study raise questions about the biological significance of these secondary compounds in tree wound repair. From macroscopic observations, the wound wood appeared to be effective in restricting the outward spread of fungal decay into subsequent functional sapwood, even 17 months after inoculation. The accumulation of

secondary compounds such as those found in the wound wood of eucalypts is generally implicated as having an antimicrobial role against pests and diseases (Woodward, 1992; Pearce, 1996). No wound is ever sterile of micro-organisms, however, given that the qualitative composition of the secondary metabolites produced in the wound wood appeared largely independent of whether or not decay symptoms were present, we propose that these chemical extractives may have a multi-functional role (not just an antimicrobial role) in eucalypt tree defence responses. Schultz and Nicholas (2000) recently found evidence to suggest that the phenolic extractives in heartwood possessed both fungicidal and antioxidant properties. Similarly, we suggest that the diverse range of secondary metabolites may all act in a synergistic manner to aid in wound healing and discourage pathogen growth in the compromised tissue. For instance, deleterious free radicals are generated from wounding (van Doorslaer *et al.*, 1999) and/or by white and brown rot fungi, which are thought to have a primary role in the depolymerisation of wood cell material (Hirano *et al.*, 1997). The secondary metabolites produced in wound wood may have an antioxidant role, acting to mop up these deleterious free radicals, particularly as both high molecular weight PAs and hydrolysable tannins have already been shown to act as strong antioxidants *in vitro* by Hagerman *et al.*, (1998) and more recently by our group (Chapter 8).

In *E. globulus* and *E. nitens*, wound wood formation appeared to consistently play an important role in the wound repair process, e.g. physically sealing off the wound site and re-establishing continuity of the vascular cambium as well as restricting the outward spread of infection into subsequent healthy tissue. Admittedly, this latter conclusion can only be drawn for the one decay species used in this study. Furthermore, an array of secondary metabolite classes were detected in the crude wound wood extracts. While the precise role of extractives in eucalypt tree defence remains unclear, the present study provides much needed background information on the chemistry of wound responses. As mentioned earlier, the chemistry of wound wood has never been analysed in other woody tree species. In future studies, it would be interesting to compare the wound wood chemistry of these other tree species with those of eucalypts. Such studies may reveal that the importance of wound wood

in tree compartmentalisation of injury and infection may have been overlooked, particularly for those species with rapid wound occlusion rates.

CHAPTER 5

Novel detection of formylated phloroglucinol compounds (FPCs) in the wound wood of *Eucalyptus globulus* and *E. nitens*

Abstract

This study characterized the chemical responses of *Eucalyptus globulus* and *Eucalyptus nitens* to artificial inoculation with a basidiomycete decay fungus. Nine-year old trees responded to mechanical wounding or inoculation with the decay fungus by producing new wound wood characterized by the presence of dark extractives 17 months after wounding. Analysis of crude wound wood extracts by HPLC coupled to negative ion electrospray mass spectrometry revealed the presence of a complex mixture of many unidentified formylated phloroglucinol compounds (FPCs), in addition to a diverse range of other polyphenolic compounds (hydrolyzable tannins, proanthocyanidins, flavanone glycosides, stilbene glycosides). Prior to this study, FPCs have only been reported from leaves and buds of *Eucalyptus* spp. Unequivocal evidence for the presence of macrocarpal A and B, and sideroxylonal A and B in the crude extracts was obtained, as well as evidence for a wide range of as yet unreported FPCs. Subsequent preliminary *in vitro* fungal and bacterial bioassays did not show that FPCs have an antimicrobial role in host-pathogen interactions in eucalypts.

5.1 INTRODUCTION

Trees respond to fungal invasion by the production of various anatomical and biochemical barriers at the lesion margin in an attempt to compartmentalize the spread of damage and decay in living sapwood (Shigo and Marx, 1977; Liese and Dujesiefken, 1990). The defence mechanisms contributing to the compartmentalization process varies between tree species, but in general, putative defence mechanisms commonly reported include the accumulation of induced inhibitory secondary metabolites (Barry *et al.*, 2001; Baum and Schwarze, 2002), the initiation of cell wall alterations e.g. suberization (Pearce, 1990) as well as occlusion of xylem cells e.g. tylosis formation within vessels (Barry *et al.*, 2000). Detailed reviews on the compartmentalization response of many woody tree species have been written by Pearce, (1996), Schwarze *et al.*, (1999) and more recently by Yamada (2001).

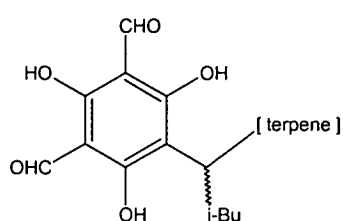
Much of the accrued knowledge concerning host-pathogens interactions in the xylem has been drawn from studies involving broad-leaved angiosperms. In comparison, the state of

knowledge on the host resistance mechanisms of *Eucalyptus* L'Heritier (Myrtaceae) is still rudimentary, despite being one the world's most important and widely planted forest species (Turnbull, 2000). In addition, the few studies that have examined host responses of *Eucalyptus* have predominantly focused on the histological aspects, and little consideration has been given to characterizing either the constitutive or induced biochemical defence mechanisms (eg. Wilkes, 1986; Barry *et al.*, 2000). Instead, chemical analyses have been generally focused on elucidating the chemistry of healthy bark (Kim *et al.*, 2000), leaves (Close *et al.*, 2001), healthy wood (Cadahia *et al.*, 1997) and kino, a polyphenolic exudate (Hillis and Yazaki, 1975). The only detailed study of the chemistry of extant tissues at wounding or infection is that reported by Barry *et al.*, (2001). Here, the decay fungi were observed to elicit increased levels of hydrolyzable tannins in the extant tissue of *E. nitens*. As part of a wider investigation examining host responses to wounding and fungal invasion in *E. globulus* and *E. nitens*, this study aimed to determine the chemical constituents present in the wound wood (defined as the tissue formed after wounding and located immediately adjacent to the wounding site). The biochemistry of wound wood has been poorly studied in both hardwoods and softwoods, and it has never been studied in any *Eucalyptus* species.

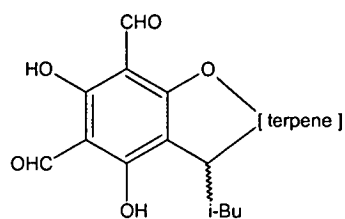
This paper will detail the detection of a group of secondary metabolites referred to as formylated phloroglucinol compounds (FPCs) in a novel location i.e. in the wound wood of *E. nitens* and *E. globulus*. We also examined the possible antimicrobial role of FPCs in host-pathogen interactions in eucalypts. FPCs are a group of secondary metabolites unique to plants in the family Myrtaceae, especially *Eucalyptus* species, and have previously been reported in buds and leaves only (Takasaki *et al.*, 1994a, 1994b; Osawa *et al.*, 1996). Detection of FPCs in wood has not been published though euglobals have been detected in *E. globulus* healthy sapwood (M. Takasaki, personal communication).

FPCs are mono- to tetra-formylated phloroglucinol based derivatives with an attached terpene moiety in almost all cases and to date, various monoterpenes and sesquiterpenes have been found linked to the formylated phloroglucinol ring. The generic structures of two

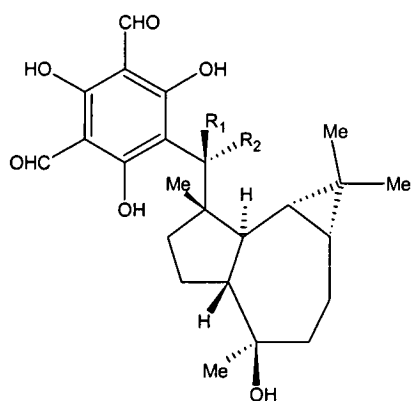
main classes of FPCs; macrocarpals and euglobals (**I** and **II**, respectively) consist of standard terpenes linked to an isopentyl diformyl phloroglucinol group. For example, Macrocarpals A and B (**III**, and **IV**) are different stereoisomers around the isobutyl group of the common sesquiterpene alcohol globulol. Other FPCs including the various known sideroxylonal isomers (Sato *et al.*, 1992) are simply two isopentyl diformyl phloroglucinol without any attached terpene group. We provide evidence for the detection of novel FPC structures including triterpene FPCs and a range of di-substituted FPCs.



(I) General structure of
macrocarpals



(II) General structure of
euglobals



(III) Macrocarpal A (R₁=H, R₂=isobutyl)

(IV) Macrocarpal B (R₁=isobutyl, R₂=H)

5.2 METHODS AND MATERIALS

5.2.1 Experimental site and wounding treatments

The experimental site growing both *E. globulus* and *E. nitens* (9-year-old) was in Lewisham, Tasmania (42°49' S, 147°37' E. 20 m a.s.l.). White *et al.*, (1998) provides details of site establishment, fertilizer addition and pest control of the Lewisham site. In late summer of 2000, six trees each of *E. nitens* and *E. globulus* were randomly selected for wounding treatments. At breast height (*ca.* 1.5 m), a 10 mm diameter hole was drilled 3 cm into the outer sapwood. A second hole with identical dimensions to the first was drilled 30 cm above the first hole and inoculated with decay fungi previously isolated from an *E. nitens* white-rot decay column. Inoculation involved inserting 2 x 1 cm² rectangular pieces of a 2-week-old culture growing on malt extract agar into the drill hole and sealing with a layer of lanolin wax. The decay fungus used for the inoculation is an unidentified basidiomycete known as 'Isolate D'. The fungus is described in Barry (2001).

Approximately three months later, three trees each of *E. nitens* and *E. globulus* were harvested. Billets of *ca.* 50 cm in length, that included the drill wounds, were dissected with a bandsaw by cutting longitudinal sections through the wound. Chisel shavings (60-90 mg) of the wound wood that had been produced adjacent to the drilling wound (Fig. 5.1), as well as samples of healthy sapwood and heartwood obtained from the same corresponding tree were prepared for LC-MS analysis as follows. The chisel shavings were extracted twice with 750 µl of 70% aqueous acetone over 48 hours in the dark at 4 °C. The extract was then transferred to a 1.5 mL microcentrifuge tube and centrifuged (1300 rpm for 2 min) to remove solids. Samples were stored at -20°C and analyzed within one week after extraction. This procedure was repeated with the remaining three trees, approximately 17 months after wounding.

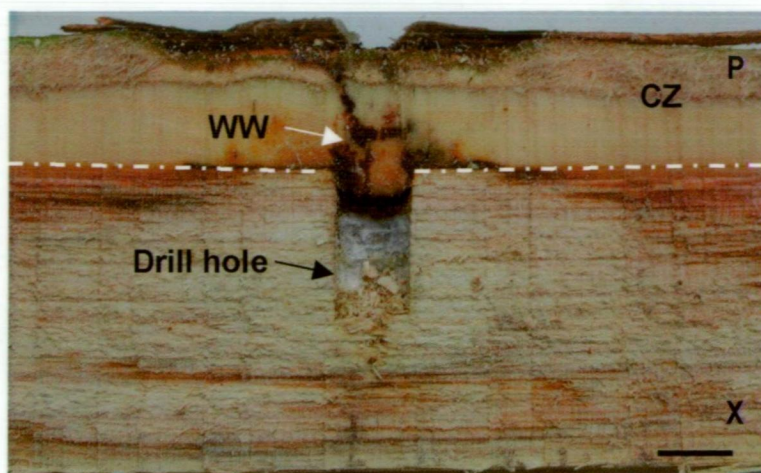


Fig. 5.1. Radial longitudinal section showing typical response to drill and inoculation treatment after 17 months in 9-year-old-*E. globulus* and *E. nitens*. Note boundary of healthy tissue and decayed tissue (dashed line). P = phloem, CZ = cambial zone, WW = wound wood, X = xylem. Bar = 1 cm.

5.2.2 Chromatographic and spectrometric analysis

HPLC-UV. Injections of 5 μL of sample were analysed by HPLC with ultraviolet detection (HPLC-UV) with the following system. HPLC analyses were carried out on a Waters Alliance 2690 with a Waters 996 Photodiode Array Detector (Waters Corporation, Milford, MA, USA). The column was a Waters Nova-Pak C18 column (150 mm \times 3.9 mm) fitted with an Alltech Econosphere 5 μm C-18 guard cartridge (Alltech, Melbourne, Australia). The flow rate was 0.8 mL min⁻¹. The solvent system was: solvent A, 2% acetic acid in methanol; solvent B, 2% acetic acid in distilled water; solvent C, 100% hexanes. The solvent gradient was as follows: 10% solvent A/90% solvent B; ramped to 85% solvent A/15% solvent B at 25 min; then to 100% solvent A at 35 min; then to 80% solvent A/20% solvent C at 45 min. The original conditions were re-established by ramping to 100% solvent A for 2 min followed by 15-min re-equilibration. The photodiode array detector was monitored from 240 to 445 nm at a sampling rate of 1 spectrum s⁻¹ and at a resolution of 2.4 nm. The HPLC sample chamber was maintained at 5 °C throughout the analyses

HPLC-MS. The HPLC column and conditions were described as above. The HPLC column outlet was coupled directly to a Finnigan LCQ ion trap mass spectrometer fitted with an electrospray source (Finnigan MAT, ThermoQuest, Co., San Jose, CA, USA). The instrument was operated in the negative ion mode, scanning from m/z 125-1500 with an automatic gain control target value of 2×10^7 and maximum ion injection time of 100 ms. Operating conditions were as follows: sheath gas 90 psi, aux gas 50 psi, ESI needle voltage 4.5 kV, capillary temperature 270 °C and capillary voltage -30 V. Data- dependent MS/MS spectra were routinely acquired from the most intense ion in the spectrum, with collision energy of 30% and a peak isolation width of 4 daltons.

5.2.3 Identification of FPCs

HPLC-ESI-MS with negative ion detection has proven to be a highly sensitive method for detection and identification of individual FPCs in a crude extract (Eschler, *et al.*, 2000; Close *et al.*, 2001). Using data from previous investigations of *E. nitens* foliage (Close *et al.* 2001), the majority of peaks within the HPLC time range 25 to 45 minutes were initially identified from their negative electrospray MS and MS/MS data as FPCs. Their identities were later confirmed with various purified FPCs standards that included euglobal Ib, euglobal Ic, euglobal III, macrocarpal A and macrocarpal B, and sideroxylonal A and B. Standards were weighed and dissolved in 100% acetone at concentrations of 0.10 mg/100 μ L. For confirmation of proposed novel triterpene FPCs and disubstituted FPCs, accurate mass measurements were carried out on a Kratos ISQ mass spectrometer fitted with a Desorption Chemical Ionisation (DCI) probe. The probe was rapidly heated to 500 °C after insertion into the ion source. The instrument was operated in the electron ionisation (EI) mode at 70 eV with an accelerating voltage of 5.3 kV. Perfluorokerosene was used as an internal reference, and peak matching was undertaken on the target ions at a resolution (10% valley definition) of 10000.

5.2.4 Semi-quantification

Total soluble extract in wound wood. To obtain the dry weights of the total soluble extractives present in the 1.5 mL solvent, 400 µL of the sample was placed into a pre-weighed glass vial (using a 5-point balance scale) then dried under a stream of nitrogen at room temperature and placed in an oven (30°C) to remove all moisture before re-weighing glass vials. Sub-sampling was necessary as the extractives could not be easily reconstituted. Typical yields of dry weight of total soluble extract (as a percentage of fresh weight of tissue) ranged from 15% to 45% for wound wood and 2% for healthy sapwood extracts.

Total levels of FPCs (% of dry weight of soluble extract). HPLC chromatograms of crude wound wood extracts showed that the separation of FPCs into individual compounds was difficult, as found in other studies (Akano *et al.*, 1981, Eschler and Foley, 1999).

Consequently, semi-quantification of individual FPCs was not attempted due to the large number of individual isomers and consequent complex chromatograms with many unresolved peaks. Instead, FPCs were measured as a single class of compounds to provide an estimate on the total levels of FPCs (expressed as % of dry weight of soluble extract) present in wound wood. The sums of the FPC peak areas (at 280 nm) as a single group were used in the semi-quantification calculations. Response factors calculated from an average of macrocarpal A and macrocarpal B standards were applied for the quantification of FPCs. The highly variable nature of wound wood as well as the small sample size precluded the use of statistical analysis to determine differences in either the qualitative and quantitative FPC composition between treatments.

5.2.5 Antimicrobial bioassays

Semi fractionation of crude wound wood extract. Semi-preparative separation of the extract was possible due to the large time interval that separated the significantly less polar fraction of the extract (predominantly containing FPCs) from the polar fraction of extract (containing the other classes of compounds). Crude wound wood extract from *E. globulus* containing relatively high FPC levels (3.4% of dry weight of soluble extract) was used. As only a crude

separation was required, a more rapid solvent system was developed. The same equipment used in the HPLC-UV analyses of the crude wound wood extract as previously described was used for the semi-preparative separation. The flow rate was 0.8 mL min⁻¹. The solvent system was: solvent A, 2% acetic acid in methanol; solvent B, 2% acetic acid in distilled water; solvent C, 100% hexanes. The solvent gradient was as follows: 75% solvent A/25% solvent B at 2 min; ramped to 100% solvent A at 4 min; then to 80% solvent A/20% solvent C at 5 min and held until 11 min. The first fraction (Fraction I) containing condensed tannins, hydrolyzable tannins and flavonoids was collected between 0-5 min. The second fraction (Fraction II), containing predominantly FPCs was collected between 5-10 min. Injections of 100 µL were made and a total of 1.6 mL was processed. All solvents collected from the semi-preparation separation were removed with a rotary evaporator at less than 40 °C. The extract was re-dissolved to original concentrations with 70 % acetone.

Antifungal Bioassay. 20 µL each of the Fraction I, Fraction II, original crude wound wood extract and a control (70% aqueous acetone) was spotted directly onto the centre of separate silica TLC plates (2 cm x 5 cm). The TLC plates were placed in laminar flow for 2 hours to remove all traces of the solvent. A dense spore and mycelium suspension of three fungal cultures (*Cladosporium cladosporioides*, *Ganoderma weberianum* and *Trametes zonata* grown on 2 % malt extract agar (Oxoid) and incubated at 20 °C in the dark) were prepared by flooding 14-day-old plates with 3 % malt extract aqueous solution and agitating the plate with a glass hockey stick to dislodge the conidia and mycelium. An even layer of the fungal suspension was applied onto the pre-prepared TLC plates. Following incubation at 22 °C under high humidity for 5 days, plates were examined for zones of inhibition.

Antibacterial bioassay. These were performed following a modified Kirby-Bauer disc diffusion procedure (Bauer *et al.*, 1966). 10 µL each of Fraction I, Fraction II, original crude wound wood extract and a control (70% aqueous acetone) was applied to individual paper discs (13mm diameter, Whatman), allowed to dry and placed on nutrient agar plates seeded

with either *Escherichia coli*, *Erwinia* sp. *Xanthomonas* sp. (Gram negative bacteria) and *Streptococcus aureus*, *Bacillus subtilis* (Gram positive bacteria). All tests were carried in triplicate and plates were incubated at 37 °C. After 48 hours incubation, the diameters of the zones of inhibition were measured.

5.3 RESULTS

Seventeen months after injury, all drill hole wounds had fully occluded and the vascular cambium had been re-established. Decay columns (>10 cm in length) were associated with all drill holes inoculated with the decay fungus. In both species, the wound wood was characterized by the presence of varying amounts of dark extractives (defined as non-structural components of wood), regardless of whether decay symptoms were evident or not (Fig. 5.1).

5.3.1 Analysis of new wound wood

A diverse range of polyphenolic compounds including hydrolyzable tannins (both gallo- and ellagi-tannins), proanthocyanidins, flavanone glycosides, stilbene glycosides (Chapter 4) and formylated phloroglucinol compounds (FPCs) were detected in the crude wound extracts of both *E. nitens* and *E. globulus*. In particular, FPCs were noted as a complex series of peaks eluting late in the HPLC chromatogram (Fig. 5.2). The presence of sideroxylonal A and B was unequivocally confirmed by comparison of HPLC-UV and MS data standards. Co-injections with FPC standards unequivocally confirmed the presence of macrocarpals A and B in the wound wood crude extract.

5.3.2 Tandem mass spectral data of macrocarpal and euglobal standards

Macrocarpals and euglobals of the same terpene series are indistinguishable by molecular weight (since they have the same degree of unsaturation despite the bond to the phenolic ring in euglobals). Tandem mass spectrometry however, provides valuable characteristic spectra to distinguish between the two FPC classes. In the present study, the sesquiterpene

macrocarpals for which standards could be obtained gave an intense product ion at m/z 207, with a weaker product ion at m/z 250 (Fig. 5.3).

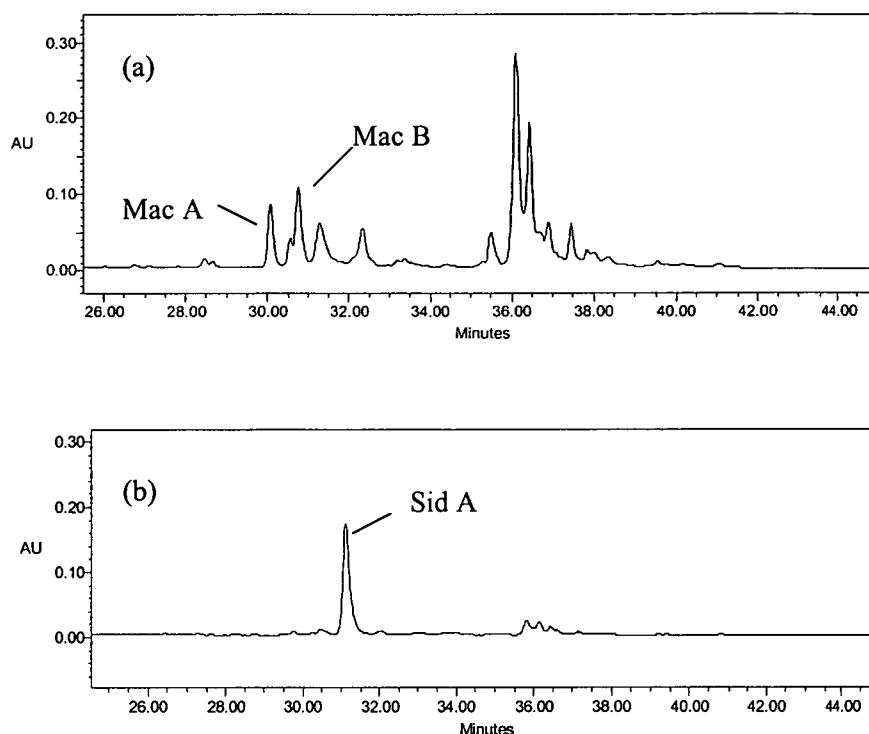


Fig. 5.2. Examples of HPLC-UV chromatograms of wound wood extract (70% acetone) of *Eucalyptus globulus* (a) and *E. nitens* (b) measured at 280 nm indicating major FPCs including sideroxylonal A (Sid A), macrocarpal A (Mac A) and macrocarpal B (Mac B).

On the other hand, euglobals Ib, Ic, III and V were characterised by an intense ion at m/z 249, and in some cases a less intense ion at m/z 207. For example, under negative ESI, the MS of Euglobal Ic gave m/z 385 (100%) while MS/MS of the m/z 385 ion showed m/z 249 (100%), 250 (11.6%) and 248 (10.6%). We suggest that the radical cation at m/z 250 in the macrocarpals can be formed by a single hydrogen transfer from the isopentyl diformyl phloroglucinol to the terpene with charge retention on the phloroglucinol ring. In the euglobals there is already one less phenolic hydrogen and a second bond has to be broken to remove the terpene group, resulting in a prominent even electron ion at m/z 249.

5.3.3 Wound wood dominated by FPCs of the macrocarpal series

Comparison of HPLC-UV and MS/MS behaviour of the FPCs present in wound wood with that described for the euglobal and macrocarpal standards indicated that the FPCs found in

wound wood were predominantly of the macrocarpal series and not of the euglobal series. More specifically, the overwhelming majority of compounds of Mol. Wt. 386, 454 and 472 gave MS/MS spectra dominated by m/z 207 and 250 product ions (Fig. 5.3). Only a few very minor peaks were characterised by an intense ion at m/z 249, consistent with their assignment as euglobals. Moreover, comparison of HPLC-UV and MS/MS data of the four available euglobal standards with the few euglobals detected in the wound wood crude extract showed the latter to be different to the euglobal standards.

5.3.4 Evidence for new FPCs

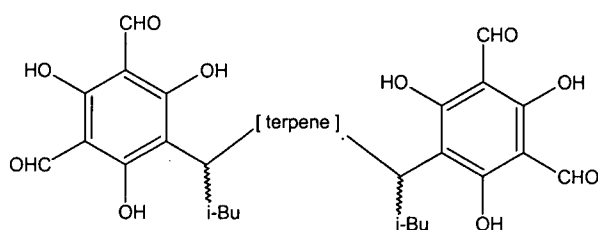
The number of co-eluting peaks detected at Mol. Wt. 386, 454 and 472 in the wound wood extracts far exceeded the number of reported compounds, indicating the presence of many new FPCs at these specific masses. In addition, complex groups of compounds of molecular weight series other than 386, 454 and 472 were also detected in the specific mass chromatograms. Significantly, tandem MS investigation of these compound series showed that they also appeared to fit the macrocarpal pattern.

For example, Figure 4 shows the MS² and MS³ experiments on a major group with intense [M-H]⁻ ions at m/z 703. The major product ion of this at m/z 453 was comparable with the [M-H]⁻ ions of the sesquiterpene hydrocarbon FPCs. Moreover, from a MS³ experiment, its subsequent third generation ions were essentially identical to the product ions of the major Mol. Wt. 454 macrocarpals present in the samples (Fig. 3b & 4c). Given that the first loss from 703 to 453 (250 daltons) corresponds in mass to an isopentyl diformyl phloroglucinol group after a single proton transfer, these compounds would appear to be the first reported terpenes with two of these groups attached (V). Accurate mass measurement by electron ionization confirmed a formula of C₄₁H₅₂O₁₀ for the Mol. Wt. of 704 (found 704.3559, calculated 704.3561).

Other new compound series with Mol. Wts. 658 and 676 were tentatively assigned as coming from triterpene hydrocarbon and triterpene alcohol FPCs respectively, also the first

report of this type of FPC (Fig. 5.5). Accurate mass measurement by electron ionization confirmed a formula of $C_{43}H_{62}O_5$ for the molecular weight of 658 (found 658.4617, calculated for 658.4598). Other series with molecular weights 590 and 608 were tentatively assigned as sesterterpene (C_{25}) hydrocarbon and sesterterpene alcohol FPCs respectively.

Further minor putative di-substituted groups were tentatively identified at molecular weights of 636, 722, 908 and 926 as monoterpene hydrocarbon bis-FPCs, sesquiterpene alcohol bis-FPCs, triterpene hydrocarbon bis-FPCs and triterpene alcohol bis-FPCs respectively on the basis of their MS/MS fragmentation behaviour. Proposed combinations of terpene and diformyl phloroglucinol moieties are listed in Table 5.1.



(V) Proposed general structure for di-substituted FPCs.

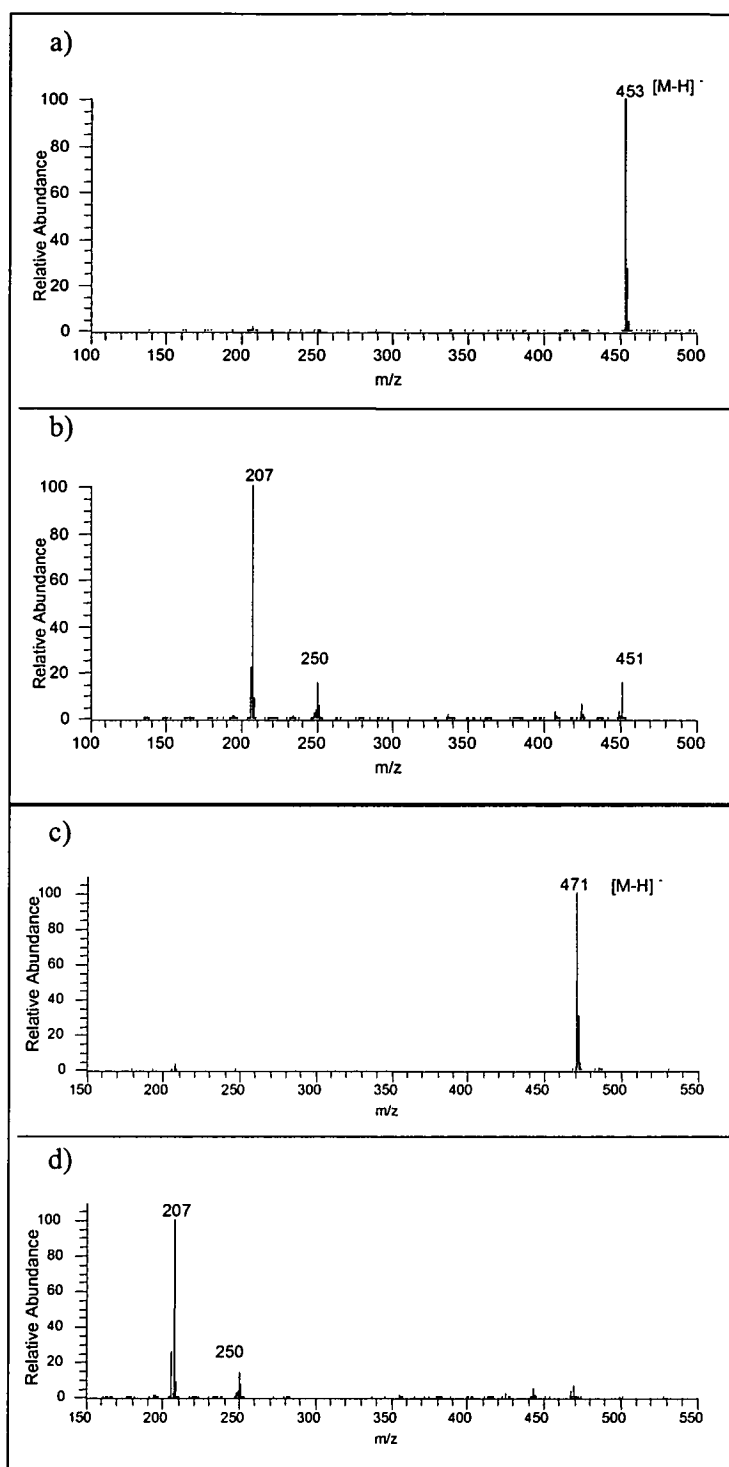


Fig. 5.3. MS and MS/MS of typical 454 sesquiterpene hydrocarbon FPC from wound wood extract and Macrocarpal A

(a). Negative ion electrospray mass spectrum of 453

(b). MS/MS product ions of [M-H]⁻ ions at m/z 453.

(c). Negative ion electrospray mass spectrum of Macrocarpal A

(d). MS/MS product ions of [M-H]⁻ ions at m/z 471 of Macrocarpal A.

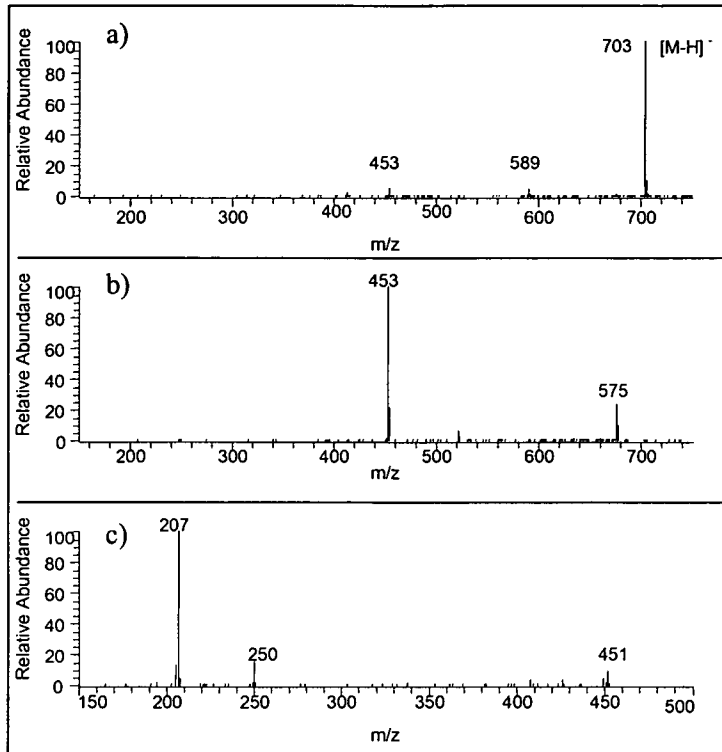


Fig. 5.4. Putative di-substituted sesquiterpene hydrocarbon FPC MS, MS² and MS³ data from *E. globulus* wound wood extract

(a). Negative ion electrospray mass spectrum

(b). MS² product ions of [M-H]⁻ ions at m/z 703

(c). MS³ product ions of m/z 453 ions derived from m/z 703.

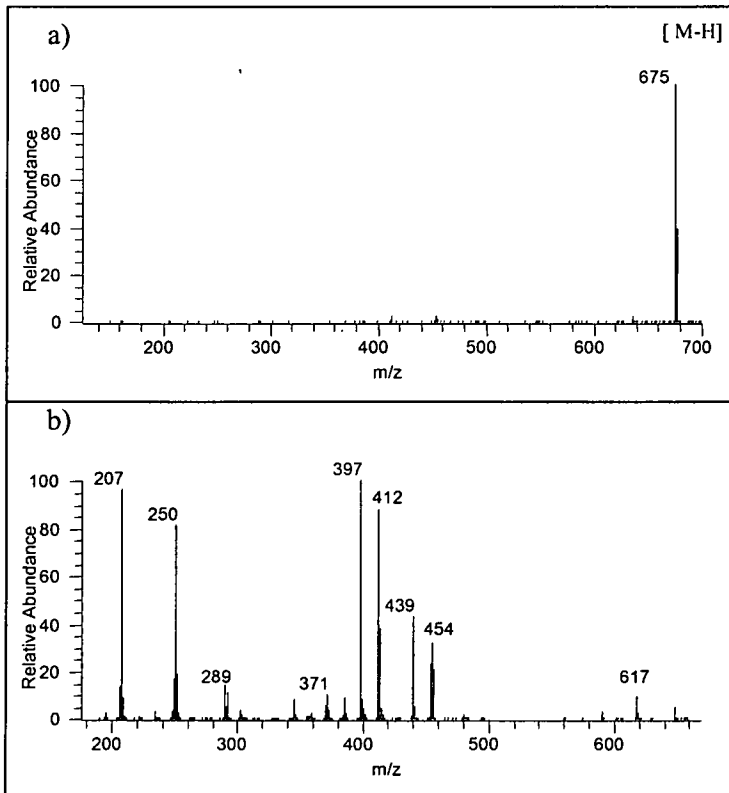


Fig. 5.5. Putative triterpene hydrocarbon FPC MS and MS/MS data from *E. globulus* wound wood extract

(a). Negative ion electrospray mass spectrum

(b). MS/MS product ions of [M-H]⁻ ions at m/z 675.

Table 5.1. List of terpene-containing FPC molecular weights and proposed terpene constituents. Previously unreported FPCs are in bold. FPCs, except those not detected (n.d), were present in wound wood

Terpene	Monosubstituted FPC	Disubstituted FPC
Monoterpene hydrocarbons	386	636
Monoterpene alcohols/ethers	404	654 (n.d)
Sesquiterpene hydrocarbons	454	704
Sesquiterpene alcohols	472	722
Sesterterpene hydrocarbons	590	840 (n.d)
Sesterterpene alcohols	608	858 (n.d)
Triterpene hydrocarbons	658	908
Triterpene alcohols	676	926

5.3.5 Semi-quantification of FPC

Total FPC content found in *E. globulus* wound wood was more variable (ranging between 0.5 to 4% of the soluble crude extract) than total FPC content present in *E. nitens* wound wood (ranging between 0.1 to 0.7% of soluble crude extract) (Table 5.2). The FPC composition of wound wood extracts obtained from inoculated treatments with that obtained from mechanical wounding only did not appear to be markedly different. A total of 14 and 13 FPC series of different Mol. Wts. were detected by MS in the wound wood crude extracts of *E. globulus* and *E. nitens*, respectively (Table 5.3). As illustrated by the HPLC-UV chromatograms (Fig. 5.2), the FPC composition of *E. globulus* wound wood was distinctly different to that of *E. nitens*. The latter species was dominated by sideroxylonal. No FPCs were found in the sapwood and heartwood of either species or in the healthy phloem of *E. nitens*. In one of two replicates of *E. globulus* healthy phloem, FPCs at relatively minor levels were detected (Table 5.3).

Table 5.2. Comparison of total FPC content in wound wood between *Eucalyptus globulus* and *E. nitens*. Mean ± SE. Sample size is shown in parentheses.

Harvest date	Species	
	E. globulus	E. nitens
3 months after treatment	0.32 * (2)	0.02 (2)
18 months after treatment	2.06 ± 0.75 (5)	0.43 ± 0.14 (4)

* weight of FPCs expressed as % of dry weight of soluble extract

Table 5.3. Comparison of the FPCs detected in wood and bark extracts of *Eucalyptus globulus* (Eg) and *E. nitens* (En) as determined by HPLC-ESI-MS.

Individual Mol Wt.	Tissue types			
	Wound Wood	Sapwood	Heartwood	Phloem
	Eg / En	Eg / En	Eg / En	Eg / En
386	+ / +	- / -	- / -	- / -
404	+ / +	- / -	- / -	- / -
454	+ / +	- / -	- / -	+ / -
500	+ / +	- / -	- / -	- / -
472	+ / +	- / -	- / -	+ / -
590	+ / +	- / -	- / -	- / -
608	+ / +	- / -	- / -	- / -
636	+ / +	- / -	- / -	- / -
658	+ / +	- / -	- / -	+ / -
676	+ / +	- / -	- / -	+ / -
704	+ / +	- / -	- / -	+ / -
722	+ / +	- / -	- / -	- / -
908	+ / +	- / -	- / -	- / -
926	+ / +	- / -	- / -	+ / -

+ detected, - not detected

5.3.6 Preliminary antimicrobial bioassays

Fungal bioassays with original crude wound wood extracts demonstrated clear inhibition of fungal growth of three species: *Cladosporium cladosporioides*, *Ganoderma weberianum* and *Trametes zonata* (Fig. 5.6). Assays with Fraction I preparations, which contained condensed and hydrolysable tannins, flavanone glycosides, and stilbene glycosides, were similarly found to inhibit fungal growth. In contrast, bioassays using Fraction II preparations, containing largely FPCs, appeared to have no antifungal effect (Table 5.4). In complimentary antibacterial bioassays, all three-fraction types inhibited the growth of Gram-positive bacteria. In these cases, antibacterial activity was most obvious in assays with original crude wound wood extracts (Table 5.5). Inhibition zones were not evident with Gram-negative bacteria (Appendix 5.1).

Table 5.4 Antifungal activity of semi-separated extracts from wound wood of *Eucalyptus globulus*.

Fungal species	Antifungal activity		
	<i>E₁</i>	<i>E₂</i>	<i>E₃</i>
<i>Cladosporium cladosporioides</i>	+	-	+
<i>Ganoderma weberianum</i>	+	-	+
<i>Trametes zonata</i>	+	-	+

E₁ = Fraction I, *E₂* = Fraction II, *E₃* = Original wound wood extract.

+ Inhibition of growth, - No inhibition of growth.

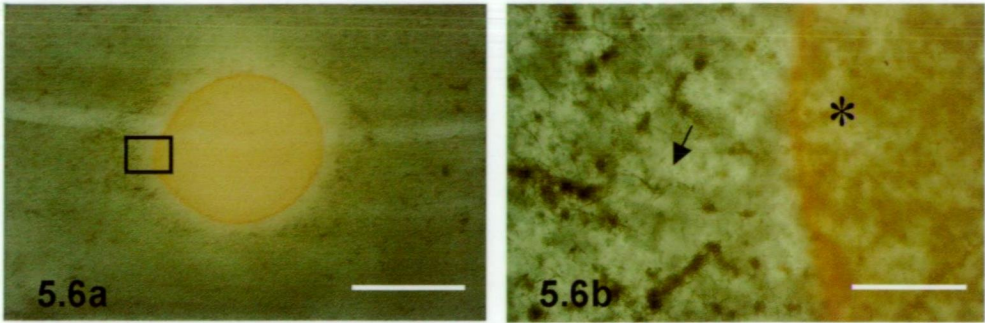


Fig. 5.6. Fungal bioassays on TLC plates showing antifungal activity of *E. globulus* original wound wood preparations against *Cladosporium cladosporioides* at low magnification (5.6a, Bar = 1 cm) and at higher magnification (5.6b is enlarged area of square in 5.6a, Bar = 0.2cm). Note interface between zone of inhibition (star) and sporulating fungal growth (arrow).

Table 5.5. Antimicrobial activity of crude wound tissue extracts from *Eucalyptus globulus*.

Microorganisms	Inhibition zone (mm)		
	E ₁	E ₂	E ₃
Gram positive			
<i>Bacillus subtilis</i>	+	+	++++
<i>Streptococcus aureus</i>	+	+	++++
Gram negative			
<i>Escherichia coli</i>	–	–	–
<i>Xanthomonas</i> sp.	–	–	–
<i>Erwinia</i> sp.	–	–	–

*E*₁ = Fraction I, *E*₂ = Fraction II, *E*₃ = Original wound wood extract.
The inhibition is reported as: (-) without inhibition, (+) *d* = 1.0-1.9 mm, (++) *d* = 2.0-2.9 mm, and (+++) *d* = 3.0-3.9 mm, (++++ *d* > 4.0 mm, where *d* is diameter of the inhibition zone.

5.4 DISCUSSION

The detection of FPCs in the wound wood of both *E. nitens* and *E. globulus* was unexpected. From macroscopic observations, the wound wood appeared to be effective at restricting the outward spread of fungal decay into new healthy tissue, even 17 months after inoculation. Therefore, we initially hypothesized that the FPCs found in wound wood might be possible inducible secondary metabolites involved in host-pathogen interactions. The accumulation of flavonoids and related polyphenols in the lesion margin of the host plant has long been

implicated in plant defence against antimicrobial invasions. Many examples of this relationship can be found in a recent review by Harborne and Williams (2000).

Subsequent antifungal bioassays conducted in this study showed original crude wound wood extract to clearly inhibit the growth of three fungal species, even using crude extracts concentrations at least ten times less than levels found *in situ* (Table 5.5). However, bioassays using preparations containing a purified FPC fraction did not inhibit fungal growth. Other workers have similarly found FPCs (macrocarpals B-G) to show no antimicrobial activity towards either yeast or fungi (Yamakoshi *et al.*, 1992). These preliminary findings indicate that the antifungal components of the wound wood are most likely found within Fraction I and future studies will aim to specifically identify these compounds.

Results obtained for the antibacterial assays were consistent with findings from other studies. As similarly found by Yamakoshi *et al.*, (1992), FPCs fractions were shown to suppress the growth of Gram-positive bacteria, eg. *S. aureus* and *B. subtilis*, but were inactive against Gram-negative bacteria. There is always a degree of uncertainty relating findings from *in vitro* bioassays to the *in vivo* situation. However, since Gram positive bacteria are not generally associated with *Eucalyptus* tree diseases (Wardlaw *et al.*, 2000), these results suggest that FPCs do not have an antibacterial role in the tree defence system or that Gram positive bacteria may not be important in diseases of eucalypts because they may be inhibited by the presence of FPCs. Interestingly, a higher level of inhibition was shown in bacterial bioassays using original crude wound wood extracts than bioassays with either extracts Fraction I and Fraction II. One possible explanation for this observation may be that during the preparation of the fractions, the volatile terpenes present in these extracts almost certainly would have been removed. Terpenoids such as those detected in wound wood extracts represent an important widespread inducible defence mechanism in Pinaceae and in particular, α -pinene has found to exhibit a high degree of biological activity towards pathogenic fungi and bark beetles (Raffa *et al.*, 1985; Delorme and Lieutier, 1990). Future

studies investigating the role of extractives present in wound wood should also aim to take into account the contribution of this group of secondary metabolites to host defence.

The role of FPCs in wound wood continues to remain unclear. The evidence is limited for a role in antimicrobial defence. To date, the chemical structures of some 50 specific macrocarpals and euglobals have been published as reviewed by Ghisalberti (1996), and Singh and Etoh (1997). We provide preliminary evidence for the discovery of many new FPCs. As mentioned earlier, FPCs have exclusively been reported in buds and leaves only. The FPCs found in leaves have been considered an important determinant governing the plant-herbivore interactions particularly of marsupial folivores. For example, macrocarpal G, jensenone and sideroxylonal were shown to be highly effective antifeedants for mammalian herbivores such as the common ringtail possum (*Pseudocheirus peregrinus*), the common brushtail possum (*Trichosurus vulpecula*) and koala (*Phascolarctos cinereus*) (Pass *et al.*, 1998; Lawler *et al.*, 1998, 1999, 2000; Staply *et al.*, 2000). Furthermore, sideroxylonal has recently been shown to have an antifeedant role against Christmas beetles (*Anoplognathus* spp.) (M. Matsuki, personal communication). From these studies examining the relationship between chemical structure and their effectiveness as marsupial antifeedants, Lawler *et al.*, (1999) proposed that there exists significant variation between different FPCs in their biological activity. If this is true, then FPCs may have multiple roles depending on composition, site of synthesis and storage. For example, FPCs found in the wound wood may be implicated in host resistance against insects as similarly found in leaves. In this case, FPCs in the wound wood may function to deter stem-boring insects from entering the stem via wound sites. Alternatively, FPCs may function as antioxidants to scavenge the deleterious free radicals generated from wounding (van Doorslaer *et al.*, 1999) and/or by white and brown rot fungi, which are thought to have a primary role in the depolymerization of wood cell material (Hirano *et al.*, 1997, Pearce, 2000). Clearly, further studies examining the role of FPCs in wound wood are required.

APPENDIX 5.1

Antibacterial bioassays on nutrient agar plates with *E. globulus* wound wood extracts.

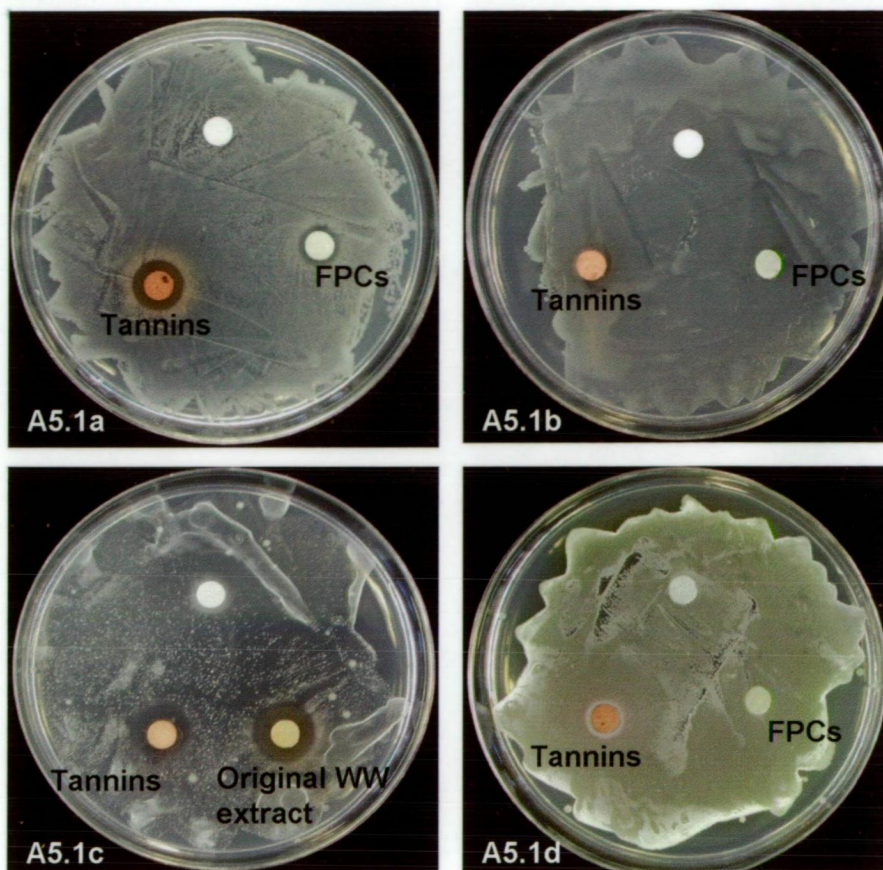


Fig. A5.1. Antibacterial bioassays following a modified Kirby-Bauer disc diffusion procedure against

- a) *Bacillus subtilis* (Gram +)
- b) *Escherichia coli* (Gram -)
- c) *Streptococcus aureus* (Gram +)
- d) *Streptomyces sp.* (Gram +)

(note: top disc in all plates is control while clear zones around disc indicate zones of inhibition).

CHAPTER 6

Traumatic oil glands induced by pruning in the wound-associated phloem of *Eucalyptus globulus*: histology and chemistry

Abstract

The natural occurrence of oil glands in various organs such as bark and leaves is well established as a characteristic of *Eucalyptus*, but this is the first reported case of traumatic oil glands induced in response to wounding. The new phloem enveloping the wound, which had developed within the two years following branch pruning in 5-year-old *Eucalyptus globulus* Labill. was morphologically distinct from healthy stem phloem. Histological examinations revealed this wound-associated phloem to be largely composed of secretory cavities similar in appearance to oil glands. Subsequent analysis of the wound-associated phloem extracts by GC-MS confirmed the presence of volatile terpenes and phenols. The total extracted oil content determined for wound-associated phloem extracts was significantly higher (>4 times) than for healthy stem phloem extracts. A comparison of the relative abundances of ten individual terpenoids from wound-associated phloem and healthy phloem revealed a number of significant differences in terpene composition. Implications of the role of terpenes as inducible secondary metabolites in tree wound responses are discussed.

6.1 INTRODUCTION

Several eucalypt species grown in Australian plantations retain rather than shed dead branches, which results in severe wood quality problems. For example, trees develop large knotty cores that make them unsuitable for the production of sawn timber and veneer (Waugh and Yang, 1994). The only way to minimize the diameter of unwanted knotty cores is by 'green pruning', the removal of live branches (Gerrand *et al.*, 1997; Mohammed *et al.*, 2000). However, pruning operations result in wounding, providing entry points for decay mainly through the branches themselves or via damage to the branch collar or stem. The occurrence of stem decay associated with pruning is high compared with unpruned trees (Mohammed *et al.*, 1998; Wardlaw and Neilsen, 1999). Elucidating the mechanisms of tree defence involved in limiting incidence and spread of decay may assist with the development of improved silvicultural practices.

In general, trees respond to wounding by the formation of both biochemical and anatomical boundaries in an attempt to compartmentalize the wounded area (Shigo and Marx, 1977). Detailed reports on the compartmentalization responses of many tree species have been extensively published as reviewed by Pearce (1996) and more recently by Yamada (2001). In contrast, the new tissue (of either the xylem or phloem) formed after wounding and located immediately adjacent to the wounding site has received relatively little attention in either hardwoods or softwoods. For example, the new phloem formed after prune wounding has never been examined in any *Eucalyptus* species. As well as compartmentalizing infections within the xylem, trees also need to close wounds to seal vulnerable entry points for decay. In the case of pruning wounds, rapid and clean occlusion of pruned stubs would appear to be essential to reduce the risk of decay entry. In some tree species, wound closure not only prevents further potential infections, but it is also able to stop subsequent fungal development in already infected wounds (Pearce, 1996). Here, complete wound closure is suggested to restrict the access of air to, and loss of moisture from compromised xylem thereby creating a microenvironment inimical to fungi colonization (Boddy and Rayner, 1983).

As part of a larger investigation comparing wounding responses between two economically important *Eucalyptus* species grown in southern Australia (*E. globulus* Labil. and *E. nitens* Dean and Maiden) (chapters 2-7), we investigated the anatomical and chemical responses of *E. globulus* to prune wounding. Preliminary investigations revealed the discovery of traumatic oil glands in the new phloem that had formed within the two years following pruning in 5-year-old *Eucalyptus globulus* plantation trees. Chemical analysis showed this new phloem to contain significantly higher total extracted oil and phenolic content than healthy phloem (Appendix 6.1 and 6.2). Oil glands do occur naturally in the plant organs of many *Eucalyptus* species including leaves, root bark, the stem pith, phloem, peduncle and floral buds, and fruit, depending on the species (Carr and Carr, 1969). Furthermore, the terpene chemistry in the aerial parts of many *Eucalyptus* species including *E. globulus* has been well studied (Boland *et al.*, 1991; Li and Madden, 1995; Li *et al.*, 1996; Bignell *et al.*,

1997). To our knowledge however, there have been no previous published reports on the occurrence of oil glands induced by wounding in the phloem of the large genus *Eucalyptus* L'Herit (>700 species).

The purpose of this study was to confirm the discovery of traumatic oil gland formation in *E. globulus* Labill. by studying the anatomical development of traumatic oil glands in wound-associated phloem and comparing the chemistry of traumatic and constitutive terpenes in response to green pruning.

6.2 MATERIALS AND METHODS

6.2.1 Experimental site and wounding treatments

Blue gum (*E. globulus* ssp. *globulus* (Labill.) of Jeeralang provenance) sample material was obtained from a 5-year-old plantation at Taranna, Tasmania (43°03' S, 147°53' E, 250 m above sea level). The long term mean annual rainfall of the study site is 900 mm. The average stand diameter and height of the trees were approximately 10.5 cm and 10.2 m, respectively. Two years prior to sampling, the trees had been pruned up to a height of 2.5 m with pruning shears as instructed by the current pruning prescription used by Tasmanian forest industry (Forestry Tasmania, 1996). The pruning aimed to remove branches flush to the stem without damaging the branch collars. Four trees of *E. globulus* excluding 'edge' trees were randomly selected for felling in late summer of 2003. For each tree, two billets of ~50 cm in length that each included approximately eleven pruning wounds were dissected with a bandsaw in the longitudinal orientation to best observe the pruning response.

6.2.2 Light microscopy

Blocks (c. 15 mm³) of the new phloem formed directly adjacent to the pruning wound (Fig. 6.1), as well as samples of healthy phloem located between pruning wounds were obtained from the same tree and fixed in FAA (formaldehyde: acetic acid: 50% ethanol; 5:5:90) for a minimum of 24 hours at 4°C, then dehydrated with an ethanol series (50%, 70%, 95%, 3 x 100%) and infiltrated in a L. R. White acrylic resin (Proscitech, Brisbane) series (30% for 24

h, 50% for 24 h, 70% for 24 h, 100% for 3 days). Samples were then polymerized in fresh 100% LR White resin for 8-10 hours at 60°C. The embedded samples were sectioned at approximately 10 µm thickness with a sledge microtome. Transverse and longitudinal sections were dried onto glass slides, stained with 1 % toluidine blue in a 0.2 M phosphate buffer (pH 6.5) and permanently mounted in Cytoseal (ProSciTech, Brisbane). Tissue structure and cell changes were observed using a Zeiss Axioskop photomicroscope.

6.2.3 GC-MS analysis

Chisel shavings (*ca.* 0.1 g) of the new phloem formed directly adjacent to the pruning wound (Fig. 6.1), as well as samples of adjacent healthy phloem obtained from the same tree were extracted twice, firstly with 2 mL of dichloromethane (containing 100 µL/ L of heptadecane as an internal standard) and then a second time with a further 0.5 mL of the same solvent over 48 hours in the dark at 4 °C. This procedure was repeated a total of three times for each of the four trees. Samples were then stored at –20°C and analyzed within one week after extraction. Analysis of the dichloromethane extracts was performed by combined gas chromatography-mass spectrometry (GC-MS) using a Hewlett Packard-5890 GC directly coupled to a Hewlett Packard 5970B Mass Selective Detector. A 25 mm x 0.3 mm diameter HP1 (0.17 µm film) fused silica GC column was used, with the oven temperature initially held at 20 °C for 2 min and then increased to 240 °C at the rate of 6 °C min⁻¹. The carrier gas was helium. Splitless injections of 1 µL were made at an injector temperature of 220 °C.

Identifications of individual compounds were based on an in-house library of reference mass spectra built up from standard compounds and oils of known composition and published Kovat's retention indices (Davies, 1990). Semi-quantification of the wet weight percentages of the total extracted oil content was determined based on the response factor of a 1,8-cineole standard ('cineole-equivalents') i.e. terpene concentrations were calculated by comparing the integrated peak area to that of the internal standard. Relative abundances of

ten selected individual oil components were determined from their percentage contribution to the total ion chromatogram (TIC).

6.2.4 Data analysis

The total extracted oil content and the percentage composition of selected terpenes extracted from healthy as well as wound-associated phloem were examined by ANOVA on SAS, Version, 8.02 following the split plot test where main plots consisted of whole trees with 6 subplots/tree, 3 of which were healthy samples and 3 were treated samples.

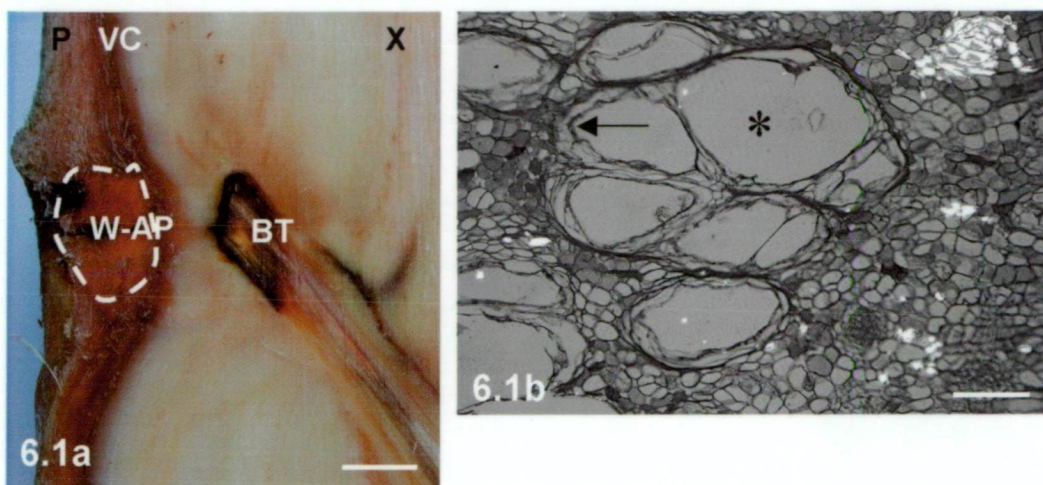


Fig. 6.1a. Radial longitudinal section showing wound-associated phloem of pruned 5-year-old *E. globulus* (dashed circle-sampling area for chemical analysis) at low magnification (a: Bar =10 mm) and at high magnification (b: Bar = 100 μ m) displaying a cluster of irregularly shaped oil glands (*) with epithelial cells (arrow). BT = branch trace, P = phloem, VC = vascular cambium, W-AP = wound-associated phloem, X = xylem.

6.3 RESULTS

6.3.1 Histology of the wound-associated phloem formed after pruning

Complete occlusion of the pruned branch in *E. globulus* had been achieved within the two years following pruning. The width of the new phloem formed directly adjacent to the pruning wound (~0.9 cm) was nearly twice that of healthy phloem (~0.5 cm) when viewed in radial longitudinal section (Fig. 6.1a). Visual assessment of the new phloem formed directly adjacent to the pruning wound suggested that it was morphologically different from healthy phloem, and microscopic examination confirmed this observation.

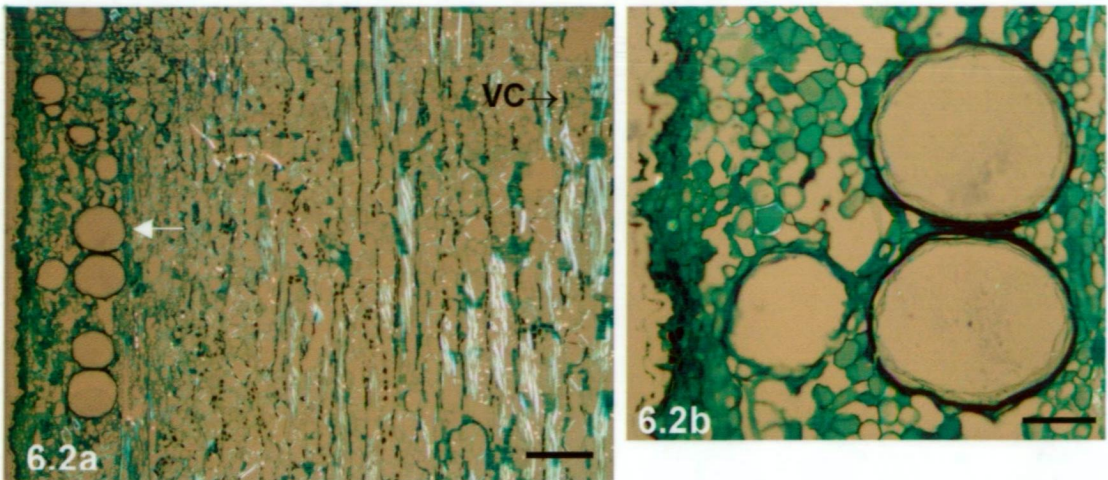


Fig. 6.2a. Radial longitudinal microtome section (toluidine blue) of healthy phloem of 5-year-old *E. globulus* at low magnification (a: Bar = 300 μm) and at high magnification (b: Bar = 100 μm), showing the spherical oil glands (arrow) in the outer cortex. VC = vascular cambium.

Healthy secondary phloem of *E. globulus* consisted of layers of phloem fibers, sieve cells, ray parenchyma and phloem parenchyma cells often containing calcium oxalate crystals (as observed under polarized light) or polyphenolic compounds. In addition, oil glands, spherical in shape and of variable sizes (ranging from 100-325 μm) were present in the outer cortex of the phloem (Fig. 6.2).

In contrast, the new phloem formed after wounding was predominately composed of irregularly shaped secretory cavities often distributed in clusters within the traumatic parenchyma (Appendix 6.3). These secretory cavities were lined with distinct epithelial cells suggesting these structures to be oil glands (Fig. 6.1b). The oil glands induced by wounding will be known as ‘traumatic oil glands’ to differentiate from oil glands naturally present in healthy phloem. Furthermore, since the new phloem that was formed after wounding was morphologically distinct from healthy phloem, the former will henceforth be referred to as ‘wound-associated phloem’.

Traumatic oil glands at varying stages of maturity were present in the wound-associated phloem making it possible to elucidate the developmental anatomy of the traumatic oil glands. From microscopic studies of different sections of the wound-associated phloem, the traumatic oil glands were shown to be produced from the de-differentiation of the traumatic parenchyma present in the wound-associated phloem. More specifically, the intracellular divisions of the traumatic parenchyma produced a group of cells characterized by the presence of dense nuclei (Fig. 6.3a). These cells break down, most likely via a schizogenous process to create the intercellular spaces, i.e. the oil cavity (Fig. 6.3b and 6.3c). In some cavities, they were bordered by a distinct layer of epithelial cells (Fig. 6.3c) while other cavities had no such epithelium but were often lined with shreds of walls from disintegrating cells. The younger stages of oil gland development were not consistently observed closer to the vascular cambium but were distributed throughout the wound-associated phloem.

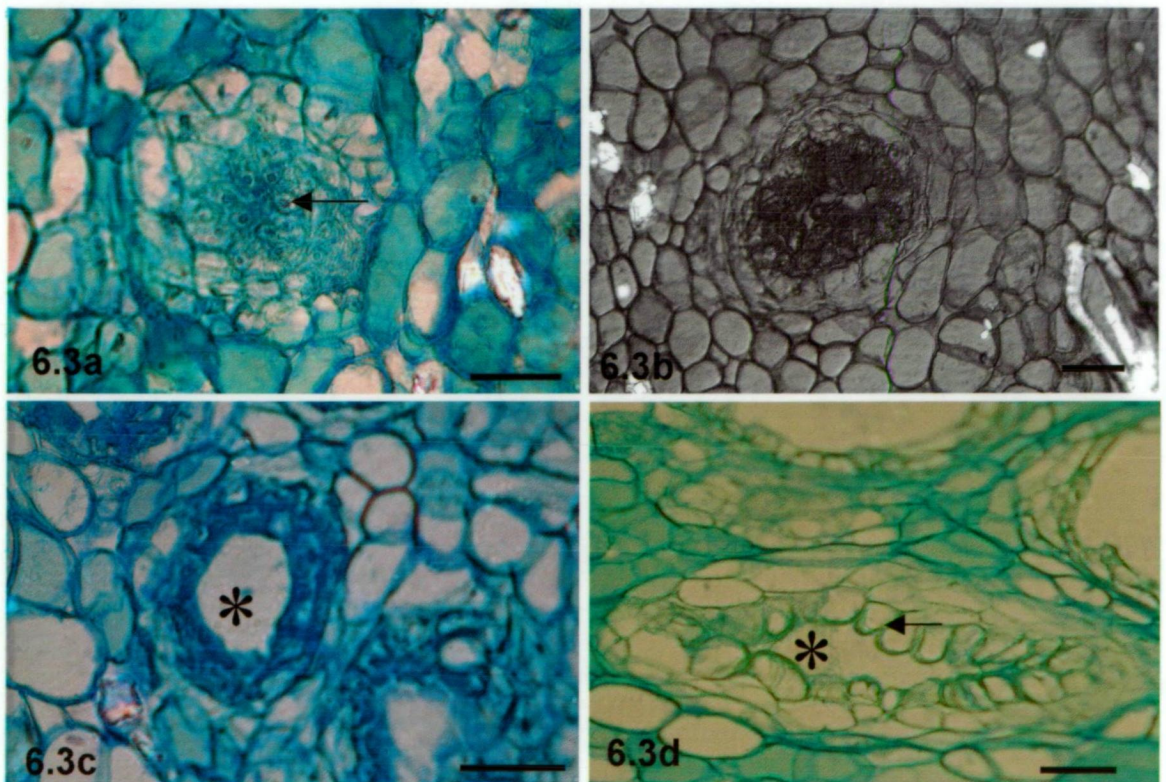


Fig. 6.3. Radial longitudinal microtome section (toluidine blue) displaying the anatomical development of traumatic oil gland formed in the wound-associated phloem of pruned 5-year-old *E. globulus*. Traumatic parenchyma subdivides to produce a group of cells containing prominent nuclei (a: arrow), which through schizogenous breakdown form an oil gland cavity (b & c *). A mature oil gland displaying epithelial cells (d: arrow) lining the oil cavity can be seen. Bar = 10 μ m (a-d).

6.3.2 Chemistry of the volatile oils detected in wound-associated phloem

The total extracted oil content in the new phloem formed subsequently to prune wounding was significantly higher than that of healthy phloem (Table 6.1). Comparisons of ten individual terpenoids from healthy phloem and wound-associated phloem revealed a number of differences in composition as listed in Table 6.2. The main terpenes detected in the wound-associated phloem were, in decreasing order of relative abundance, α-phellandrene (2), aromadendrene (7), 1,8- cineole (4) and α-pinene (1). In contrast, healthy phloem tissue comprised largely of aromadendrene (7), α-phellandrene (2), 1,8-cineole (4) and α-pinene (1), in decreasing order of relative abundance. Significantly greater amounts of α-pinene (1), terpinyl acetate (5), α-gurjunene (6) and viridiflorene (9) were present in wound-associated phloem than healthy phloem. Conversely, significantly lower concentrations of p-cymene (3) and aromadendrene (7) were detected in traumatic phloem compared to healthy phloem.

Table 6.1. Total extracted oil yield in the dichloromethane extracts of pruned 5-year-old *E.globulus*. Mean ± SE. Sample size is shown in parentheses.

Tissue type	total oil content (wet weight %) ^A
Healthy phloem	0.57 % ± 0.07 (n = 12)
Wound-associated phloem	2.53 % ± 0.36 (n = 12)

^A Total oil contents (as % of wet weight) are expressed as ‘cineole equivalents’.

Significant at P<0.0001

Table 6.2. Compounds identified and their relative percentage contribution to the TIC in wound-associated phloem extracts of pruned 5-year-old *E. globulus*. Mean \pm SE

No.	Oil compounds	Tissue type [^]		
		Healthy phloem	Wound-associated phloem*	Significance
1	α -pinene	11.8 \pm 1.2	14.7 \pm 1.7	*
2	α -phellandrene	19.3 \pm 2.1	16.9 \pm 1.4	ns
3	p-cymene	4.0 \pm 0.5	2.5 \pm 0.4	**
4	1,8-cineole	12.1 \pm 2.0	14.2 \pm 2.3	ns
5	terpinyl acetate	1.1 \pm 0.2	2.2 \pm 0.4	**
6	α -gurjunene	2.9 \pm 0.3	3.7 \pm 0.1	**
7	aromadendrene	20.7 \pm 1.7	16.0 \pm 1.8	***
8	alloaromadendrene	2.6 \pm 0.2	2.5 \pm 0.2	ns
9	viridiflorene	3.8 \pm 0.4	5.3 \pm 0.3	***
10	globulol	7.8 \pm 0.8	6.8 \pm 0.9	ns

[^] Percentage of compounds based on an average of 12 replicates. ns = not significant. *Significant at $P < 0.05$. ** Significant at $P < 0.01$. *** Significant at $P < 0.001$.

6.4 DISCUSSION

In *E. globulus*, the new tissue involved in the occlusion of the pruning wound was found to be both anatomically and chemically different to healthy phloem. The wound-associated phloem was characterized by the abundant presence of traumatic oil glands and subsequent analyses of this tissue showed it to contain not only significantly greater concentrations of volatile terpenes but also of a different composition compared to that of healthy phloem. Microscopic examinations demonstrated that the formation of traumatic oil glands appeared to follow a similar developmental process to that reported by Chattaway (1955) for oil glands in the bark of eucalypts. Chattaway (1955) found that these oil glands developed from the de-differentiation of ray parenchyma cells and not from the initials of the vascular cambium. Likewise, traumatic oil glands did not develop from the meristematic activity of the vascular cambium but from the intracellular divisions of the traumatic parenchyma of the wound-associated phloem.

The detection of traumatic oil glands in the phloem is a novel finding for eucalypts. However, the induction of specialized secretory structures that are involved in the synthesis and accumulation of terpenoid secondary metabolites (Gershenzon and Croteau, 1991) has been reported in other woody tree species. For example, the developmental anatomy, chemistry and physiology of induced specialized secretory structures known as resin cysts, blisters and ducts has been extensively studied in Pinaceae, particularly in response to bark beetle attacks and their associated pathogenic fungi as recently reviewed by Lieutier (2002). However, in the developmental anatomy studies, regardless of where the specialized secretory structures were induced in secondary xylem or phloem, they were reported to either develop from the hypertrophic dedifferentiation of extant tissue (Yamanaka, 1989) or from the meristematic activity of the vascular cambium (Babu *et al.*, 1987) at the time of wounding or treatment. In the case of eucalypts, the traumatic oil glands were detected in the new wound tissue formed sometime after wounding that was later included in the phloem. With the exception of a few studies that have reported the anatomy of wound tissue included in the xylem (Torelli *et al.*, 1994; Smith and Sutherland, 1999; Eyles *et al.*, 2003a-b), the new phloem tissue enclosing the pruning wound to our knowledge has not been previously examined for other woody tree species. Therefore it is not possible to compare the wound-associated phloem of eucalypts with that of other genera.

Considering that healthy phloem provides an important structural and chemical barrier against insect and/or fungal pathogens (Biggs, 1992), the presence of traumatic oil glands in the wound-associated phloem raises questions about its function in eucalypt wound repair and defence. The terpenoid secondary metabolites are synthesized in specialized secretory structures such as resin ducts and oil glands formed in conifers and eucalypts, respectively. Terpenoids have long been implicated in the protection of plants against herbivore attack, regardless of whether they are synthesised in constitutive or induced secretory structures (e.g. Croteau and Johnson, 1985). More specifically, both qualitative and quantitative changes are reported to occur in terpene composition after wounding and/or to insect and fungal attack in several conifers (Delorme and Lieutier, 1990; Klepzig *et al.*, 1995; Nault and

Alfaro, 2001). For example, monoterpenes (including α -pinene, β -pinene, limonene, myrcene, α -phellandrene and δ -3-carene) have been shown to accumulate in high concentrations as part of a characteristic defense response in *Pinus contorta* (Raffa and Berryman, 1982) and *Picea abies* (Baier *et al.*, 2002). Furthermore, monoterpenes such as α -pinene have been found to exhibit a high degree of toxicity towards pathogenic fungi and bark beetles from *in vitro* assays (Raffa *et al.*, 1985; Delorme and Lieutier, 1990). In the present study, the traumatic oil glands in the wound-associated phloem of *E. globulus* were found to contain a variety of monoterpenes including α -pinene and α -phellandrene at increased concentrations (Table 6.1). We suggest that the highly concentrated amounts of volatile terpenes produced in the wound-associated phloem of eucalypts may have a similar protective function as that shown for the terpenes produced in the resin ducts of many conifers.

This study investigated tree responses to only one type of mechanical wounding i.e. pruning. However, the presence of traumatic oil glands has been also recently observed in the wound-associated phloem related to fungal canker infections (Chapter 7) and stem wounds (Appendix 6.2). Therefore, it is likely that the formation of wound-associated phloem is a common and general response to all types of wounding (whether mechanical or biological) to the phloem. The induced response of traumatic oil gland formation appears to be strongly evident in older eucalypts. The presence of constitutive oil glands in the phloem has been reported to vary depending on species and age of tree (Carr and Carr, 1969). Here, these workers reported constitutive oil glands in the phloem of young *E. nitens* trees but not in mature trees. Similarly, in the current study, constitutive oil glands were detected in the healthy phloem of young *E. globulus* trees (5-year-old). However, from preliminary microscopic examinations, no constitutive oil glands were detected in the healthy phloem of mature *E. globulus* (9-year-old) plantation trees (personal observation), suggesting that the induction of traumatic oil glands may be more evident in mature trees.

. From an industry perspective, the ability of eucalypts to produce wound-associated phloem may prove to be an important inducible resistance mechanism, particularly for plantations intensively managed for solid wood products where pruning is a prescribed practice. In the search to find tree resistance mechanisms against those biological agents that reduce wood quality such as stem canker causing fungi and insect stem borers, characterizing the terpene composition of the wound-associated phloem may provide a basis for identifying susceptible or resistant species for incorporation in genetic improvement strategies.

APPENDIX 6.1**Preliminary findings: terpene content and composition of wound-associated phloem from pruned 5-year-old *E. globulus* plantation trees grown in Legerwood, Tasmania.****Table A6.1a Total oil yields in the dichloromethane extracts of pruned 5-year-old *E. globulus*. Mean \pm SE. Sample size is shown in parentheses.**

Tissue type	total oil content (wet weight %) ^A
Healthy phloem	0.04 % \pm 0.02 (n = 4)
Wound-associated phloem	2.2 % \pm 0.4 (n = 6)

^A Total oil contents (as % of wet weight) are expressed as 'cineole equivalents'.

Table A6. 1b Compounds identified and their relative percentage contribution to the total ion chromatogram (TIC) (>0.70%) in wound-associated phloem extracts* of pruned 5-year-old *E. globulus* from two sites (I, II). Mean \pm SE.

No.	Oil compounds	Site 1	Site II
		α -phellandrene type	α -pinene type
1	α -pinene	10.8 \pm 0.4	20.9 \pm 1.6
2	α -phellandrene	24.4 \pm 1.7	n.d. ^A
3	myrcene	0.6 \pm 0.	n.d
3	p-cymene	2.2 \pm 0.8	0.6 \pm 0.6
4	1,8-cineole	6.5 \pm 0.6	18.7 \pm 0.7
5	limonene	1.1 \pm 0.1	2.1 \pm 0.03
6	γ -terpinene	0.4 \pm 0.1	1.3 \pm 0.6
7	terpinolene	1.0 \pm 0.	0.4 \pm 0
8	terpinyl acetate	n.d	1.7 \pm 0.9
9	α -gurjunene	5.2 \pm 0.4 [#]	
10	β -gurjunene	1.1 \pm 0.1	
11	aromadendrene	16.0 \pm 0.8	
12	calamenene	0.7 \pm 0.05	
13	alloaromadendrene	2.7 \pm 0.06	
15	viridiflorene	6.0 \pm 0.2	
17	ledol	1.8 \pm 0.07	
18	globulol	7.8 \pm 0.3	
19	viridiflorol	1.4 \pm 0.03	
21	rosifoliol	0.8 \pm 0.02	
22	β -eudesmol	0.8 \pm 0.02	

*Percentage of compounds 1-8 based on average of 3 replicates and compounds 9-26 based on 6 replicates of wound-associated phloem extracts. ^A n.d. = not detected. [#] Compounds 9-26 are present in both types of extracts.

APPENDIX 6.2

HPLC chromatogram of crude extracts from the wound-associated phloem of pruned 5-year-old *E. globulus* trees

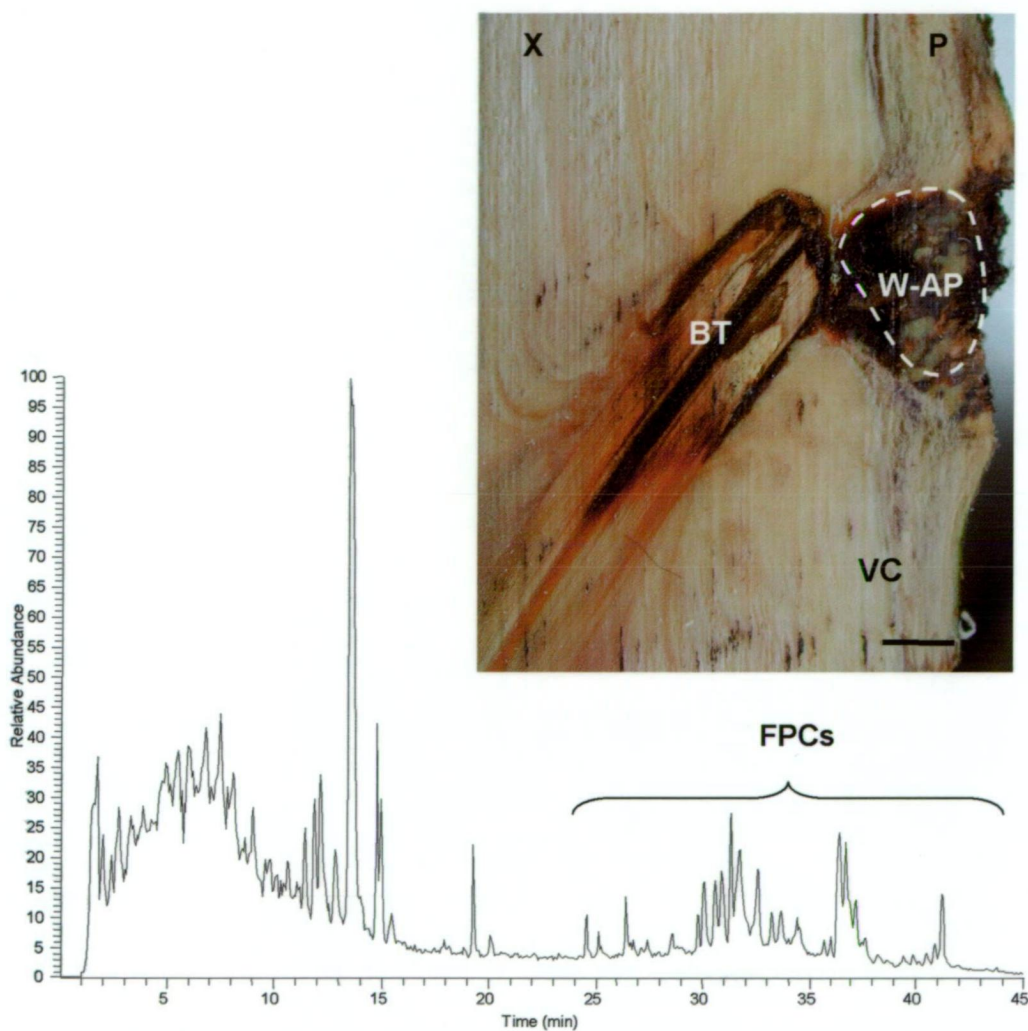


Fig. A6.2. HPLC chromatogram of 70% aqueous acetone extracts obtained from the wound-associated phloem (see macrograph – dashed circle) of pruned 5-year-old *E. globulus*. Note: HPLC chromatogram is very similar to those obtained for wound wood (Chapter 4) i.e. the unresolved peaks between 0 and 20 min, largely contain (proanthocyanidins) PAs and hydrolyzable tannins. The peaks between 25- 45 min (within bracket) largely contains formylated phloroglucinol compounds (FPCs). Bar ~ 1 cm. BT = branch trace, P= phloem, VC = vascular cambium, W-AP = wound-associated phloem, X = xylem.

APPENDIX 6.3

Anatomy of wound-associated phloem (overview).

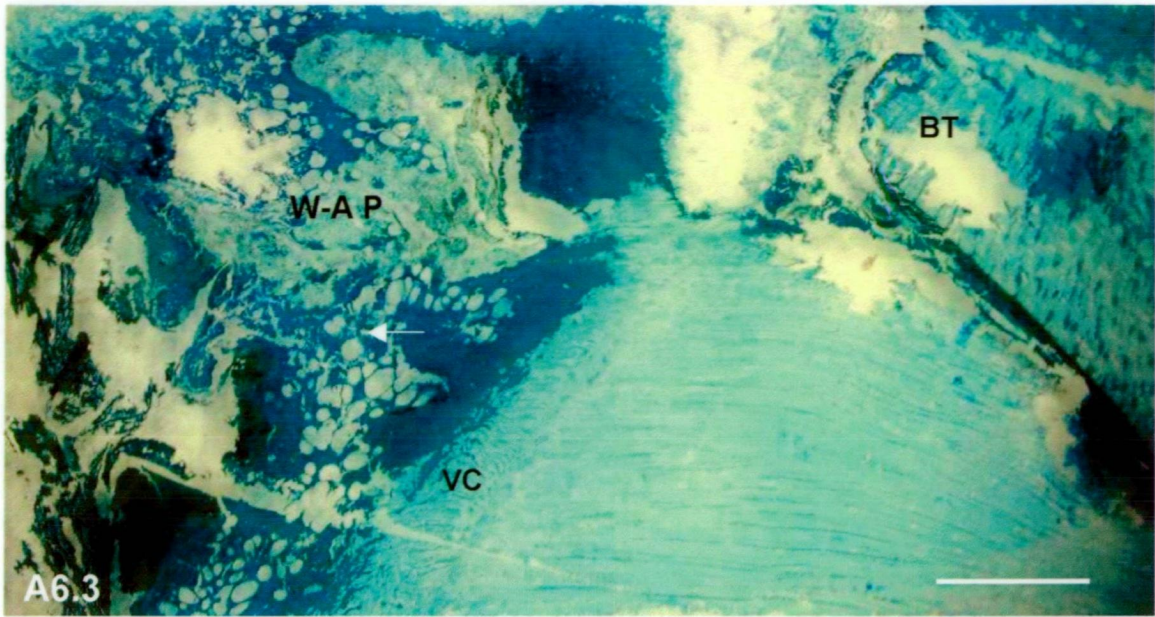


Fig. A6.3. Microtome section (toluidine blue, radial) of *E. globulus* displaying the wound-associated phloem 2 years after pruning with prolific production of oil glands (arrow). Bar ~ 1 cm. BT = branch trace, VC = vascular cambium, W-A P = wound-associated phloem.

APPENDIX 6.4

Traumatic oil glands observed in the phloem of *E. globulus* after stem wounding (cause of wound unknown).

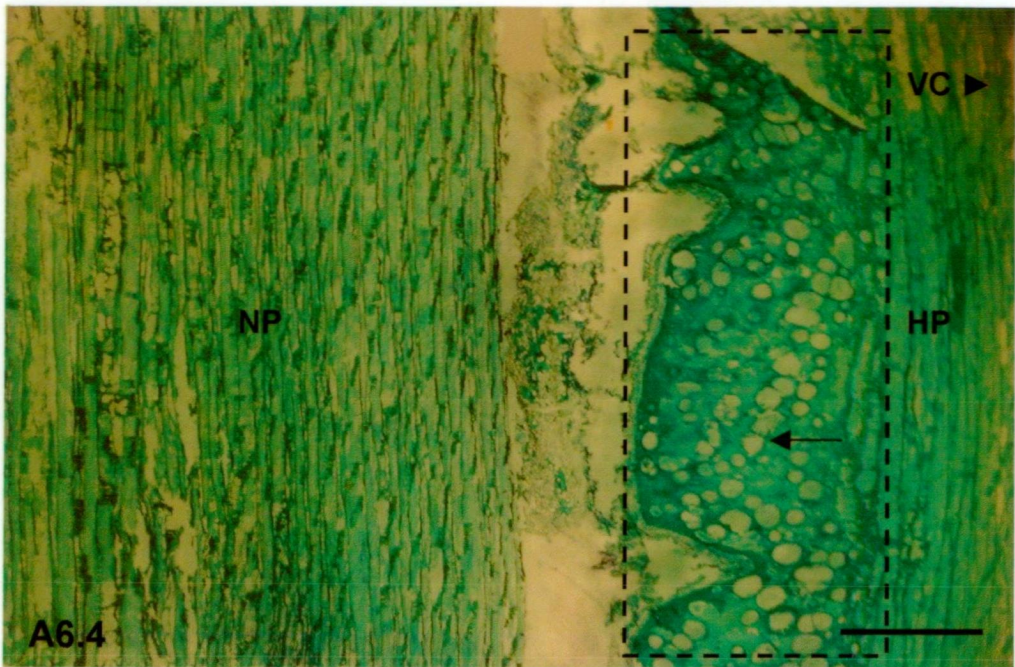


Fig. A6.4. Microtome section (toluidine blue, radial longitudinal) of *E. globulus* displaying prolific production of traumatic oil glands within traumatic parenchyma (dashed square) in the phloem of 9 year-old *E. globulus* after stem wounding. Bar = 3 mm. HP = healthy phloem, NP = necrotic phloem, VC = vascular cambium.

CHAPTER 7

Host responses to natural infection by *Cytonaema* sp. in the aerial bark of *Eucalyptus globulus*

Abstract

The chemical and anatomical host responses to natural fungal infection by *Cytonaema* sp. in the aerial bark of 3-year-old *E. globulus* plantation trees were examined. The lesion margin (LM) of the canker-infected bark was characterized by the formation of a layer of dark extractives visible to the naked eye. Chemical analysis of the LM by GC-MS, HPLC-UV and HPLC-MS using negative ion electrospray ionisation indicated the presence of a range of compounds including hydrolyzable tannins, polymeric proanthocyanidins, flavonoid glycosides, formylated phloroglucinol compounds and volatile terpenes. These compounds were either undetectable in healthy tissue or present at significantly lower concentrations than in the LM. The LM of the canker-infected bark was morphologically distinct from healthy phloem, its characteristics varying depending on severity of canker infection. In superficial infections in which only the phloem was affected, the following LMs were observed, i) a continuous wound periderm of multiple layers, or ii) an incompletely differentiated and discontinuous wound periderm. In cases of severe canker infections in which the vascular cambium had been killed, the new phloem formed subsequently contained traumatic oil glands in addition to the responses observed for superficial canker infections. All LMs were characterized by the formation of new parenchyma cells that stained positive for the presence of polyphenols. The significance of the chemical and structural responses as defence mechanisms against fungi causing stem canker is discussed.

7.1 INTRODUCTION

Eucalypts are one of the world's most important and widely planted forest species (Turnbull, 2000). Of the estimated 187 million hectares of forest plantations world wide in 2000, twenty percent were *Pinus* spp. and ten percent were *Eucalyptus* spp. plantations (Anon, 2001). However, in comparison with *Pinus* spp. the background knowledge on host responses of bark to localized bark lesions (cankers), is very limited in this large genus of *Eucalyptus* L'Herit (>700 species). The two pathogens responsible for the most severe canker diseases of eucalypt plantations around the world are caused by *Erthricium* (Corticium) *salmonicolor* and *Cryphonectria cubensis* (Bruner) Hodges (Old and Davidson, 2000; Wingfield *et al.*, 2001). In Australia, canker fungi are generally recognised as weak or opportunistic pathogens in natural eucalypts forests (Old *et al.*, 1986; Yuan and Mohammed, 1999). One exception was the severe outbreak of *Endothia gyrosa* (Schwein.: Fr) Fr in a 16-

year-old Tasmanian *E. nitens* (Deane and Maiden) Maiden plantation observed between 1993 and 1995. The fungus was reported to behave as a primary pathogen causing severe stem canker that resulted in a significant level of tree mortality (Wardlaw, 1999). A survey of Tasmanian eucalypt plantations and regrowth forests found the two most common stem fungi associated with canker diseases were *Endothia gyrosa* and *Cytospora eucalypticola* van der Westhuizen (Yuan and Mohammed, 1997). Eucalypt plantations are increasingly being planted for solid wood and veneer production instead of pulpwood in Tasmania (Gerrand *et al.*, 1997). However, the high costs of plantation management for sawlogs means that agents such as canker and wood-rotting fungi, which result in stem defect, could have a significant negative impact on the profitability of solid wood production. A better understanding of host response to stem canker infections may offer opportunities for selection of resistant individuals.

Bark provides an important structural and chemical barrier, and few pathogenic fungi are able to directly penetrate intact bark, the majority requiring wounds to facilitate their entry (Woodward, 1992; Woodward and Pocock, 1996). The induced host response to wounding in bark is non-specific; minimizing the spread of infection as well as reducing desiccation. This process involves the formation of a ligno-suberized boundary zone in cells present at the time of wounding while underlying healthy bark tissue near the lesion margins de-differentiates to form a wound periderm (WP) (Mullick, 1977; Woodward and Pearce, 1988a; Biggs, 1992; Solla *et al.*, 2002). In addition to forming these structural boundaries, secondary metabolites i.e. phenolic and terpenoid compounds have been reported to accumulate in the lesion margin (LM), particularly in gymnosperms (Woodward and Pearce, 1988b; Klepzig *et al.*, 1995; Virri *et al.*, 2001). For the broad-leaved angiosperms however, studies of the host responses to canker infection have predominantly focused on the histological aspects of bark resistance while the chemistry of wound responses in bark has received little consideration. This has also been true for the few studies that have investigated the host responses of bark to fungal infections in *Eucalyptus* (Tippett *et al.*, 1983; Tippett and Hill, 1984; Wardlaw, 1999; Yuan and Mohammed, 2001). Until recently,

chemical analyses of this relatively large genus have generally focused on determining the chemistry of healthy bark (Cadahia *et al.*, 1997a; Lee *et al.*, 1998; Kim *et al.*, 2000), leaves (Boland *et al.*, 1991; Close *et al.*, 2001), healthy wood (Cadahia *et al.*, 1997b) and kino, a polyphenolic exudate (Hillis and Carle, 1962; Hillis and Yazaki, 1975). The chemical composition of bark in response to stem canker infection has never been previously published for any *Eucalyptus* species. The main objective of this study was to describe both the anatomical and chemical responses to natural infection by a stem canker fungus (*Cytonaema* sp.) Höhn in the aerial bark of 3-year-old *Eucalyptus globulus*. *Cytonaema* sp. has not previously been a recognized pathogen associated with stem cankers of eucalypts in Tasmania (Yuan and Mohammed, 1997). Results from preliminary inoculation tests provided evidence to suggest that this fungus does have the ability to cause necrotic lesions around cambial wounds following artificial inoculation (Appendix 7.1).

7.2 MATERIALS AND METHODS

7.2.1 Sample collection

During early summer of 2002, billets of *ca.* 50 cm in length, that included the bark of four randomly chosen 3-year-old *Eucalyptus globulus* trees naturally infected with *Cytonaema* sp. (Fig. 7.1) were collected from a plantation site located in Taranna, Tasmania (41°15' S, 147°41' E, 450 m a.s.l). Numerous sunken discrete necrotic lesions of an elliptical shape, approximately 1.5 cm long and 1 cm wide (Fig. 7.1) were evident on the stems of *E. globulus* trees. The long-term mean rainfall of the plantation is 990 mm pa. The average stand diameter and height of the trees were approximately 6 cm and 3.6 m, respectively. Infected stems were dissected longitudinally through the lesion with a bandsaw. Both *E. globulus* and *E. nitens* were planted on the same site, but necrotic lesions were not observed on the bark of any *E. nitens* trees. The development of stem cankers did not appear to be associated with any obvious stem wounds or environmental stress. Conidiomata and micro-conidia, characteristic of *Cytonaema* sp. were produced abundantly on the surface of some cankers (Fig. 7.2a and 7.2b, respectively). The eustromatic conidiomata were slightly immersed in the bark, pyriform with the neck attenuated and blackened (Fig. 7.2a).

Longitudinal dissections of the canker infection revealed that in severe cases, the vascular cambium had been killed (Fig. 7.3a) while in others, only the phloem layer had been affected. Furthermore, in some cases, the cankers infections were observed to be associated with the presence of a continuous layer of dark extractives (visible to the naked eye) along the interface between infected necrotic and healthy phloem tissues (Fig. 7.3a). Pure cultures of *Cytospora* sp. were isolated from small samples of sterilized bark supporting pycnidia grown on 2 % malt extract agar (MEA).

7.2.3 Preparation of tissues for histological examination

Blocks (c. 15 mm³) including the lesion margin and healthy phloem were fixed in FAA (formaldehyde: acetic acid: 50% ethanol, 5:5: 90 v/v/v) for a minimum of 24 hours at 4°C, dehydrated with an ethanol series and infiltrated in a LR White acrylic resin (Proscitech, Brisbane) series. Samples were then polymerized in fresh 100% LR White resin for 8-10 hours at 60°C (Chapter 2). Transverse and longitudinal sections of 10 µm in thickness were cut with a sledge microtome, dried onto glass slides, stained with a 1 % aqueous solution of toluidine blue and permanently mounted in Cytoseal (ProSciTech, Brisbane). Tissue structure and cell changes were noted on a Zeiss Axioskop photomicroscope (Germany). Lignin was detected with phloroglucinol-HCl (Jensen, 1962) while suberin was detected with Sudan Black B (Jensen, 1962) and the autofluorescence quenching technique (Biggs, 1984). Autofluorescence of lignin was quenched with 1 % phloroglucinol-HCl and the residual distinct blue autofluorescence was observed under a Zeiss Axiovert 35 fluorescence microscope (Germany) equipped with a HBO 50-W mercury lamp with BP 340-380 excitation and LP 425 barrier filters. Monomeric flavan-3-ols as well as the corresponding oligomeric proanthocyanidins were detected using *p*-dimethylamino-cinnamaldehyde (DMACA) with a method modified from Gutmann (1993) described elsewhere (Chapter 2).

7.2.2 Chromatographic and spectrometric analysis

High performance liquid chromatography with ultraviolet detection (HPLC-UV) and high performance liquid chromatography coupled with electrospray ionization mass spectrometry (HPLC-MS) analyses of extracts were restricted to those canker infections that were associated with the production of macroscopically obvious dark extractive as part of the lesion margin (Fig. 7.3a). Chisel shavings of bark naturally infected with canker as well as samples of healthy bark and sapwood obtained from the same tree (80-100 mg) were extracted twice with 750 μ l of 70% aqueous acetone over 48 hours in the dark at 4 °C. The extract was then transferred to a 1.5 mL microcentrifuge tube and centrifuged (at $g = 0.42$ for 2 min) to remove solids. This procedure was repeated for each of the four trees. Terpene analysis using combined gas chromatography-mass spectrometry (GC-MS) was carried out for those severe canker infections in which the vascular cambium had been previously killed. Chisel shavings of bark naturally infected with canker as well as samples of healthy bark obtained from the same tree (100-120 mg) were extracted with 2 mL of dichloromethane over 24 hours in the dark at 4 °C. A total of seven samples were obtained from severe canker infections and four from healthy phloem. For both HPLC-MS and GC-MS analyses, samples were stored at -20°C and analyzed within one week after extraction.

HPLC-UV and HPLC-MS analysis: Analyses of samples (5 μ L per injection) were carried out on a Waters Alliance 2690 HPLC with a Waters 996 Photodiode Array Detector (Waters Corporation, Milford, MA, USA). The column was a Waters Nova-Pak C18 column (150 mm x 3.9 mm) fitted with an Alltech Econosphere 5 μ m C-18 guard cartridge (Alltech, Melbourne, Australia). The HPLC column outlet was coupled directly to a Finnigan LCQ ion trap mass spectrometer fitted with an electrospray source (Finnigan MAT, ThermoQuest, Co., San Jose, CA, USA). The instrument was operated in the negative ion mode, scanning from m/z 125-1500 with an automatic gain control target value of 2×10^7 and maximum ion injection time of 100 ms. Detailed descriptions of the operating conditions for both HPLC-UV and HPLC-MS can be found in Chapters 4 and 5.

The identification of compound classes and specific compounds within them was achieved by a combination of interpretation of mass spectrometry (MS) and tandem MS data from first principles, UV spectra, and comparison of HPLC, UV and MS data with standards where possible (De Pascual-Teresa *et al.*, 1998; Hammerstone *et al.*, 1998; Stobiecki *et al.*, 2000; Barry *et al.*, 2001; Davies and Barry, 2001; Chapters 4 and 5). For simplicity, semi-quantification was restricted to 3 single compounds (catechin, engelitin and pedunculagin), and 2 class compounds (polymeric proanthocyanidins (PAs) and formylated phloroglucinol compounds (FPCs)). Calculations of the percentage wet weights of the single and class compounds were based on the total absorbance at 280 nm of the HPLC-UV chromatogram, with some assignment in regions of co-elution, referred back to individual MS responses. Correction factors calculated from pedunculagin, macrocarpal A and (+)-catechin standards were applied for the semi quantification of pedunculagin, FPCs and catechin, respectively. An estimation of total polymeric PAs was based on the response factor of a catechin standard ('catechin equivalents'). For engelitin, calculations were based on its molar absorbtivity (15849, Hillis and Carle, 1962) at 294 nm.

GC-MS analysis: Analysis of the dichloromethane extracts was performed by combined gas chromatography-mass spectrometry (GC-MS) using a Hewlett Packard-5890 GC directly coupled to a Hewlett Packard 5970B Mass Selective Detector, Palo Alto, California. A 25 mm x 0.3 mm diameter HP1 (0.17 μ m film) fused silica GC column was used, with the oven temperature initially held at 20 °C for 2 min rising by 6 °C min⁻¹ to a final temperature of 240 °C. The carrier gas was helium. Splitless injections of 1 μ L were made at an injector temperature of 220 °C. Identifications of individual compounds were based on an in-house library of reference mass spectra built up from standard compounds and oils of known composition and published Kovat's retention indices (Davies, 1990). Semi-quantification of the wet weight percentages of the total oil content was determined based on the response factor of a 1,8-cineole standard ('cineole-equivalents'). Relative abundances of individual oil components were determined from their percentage contribution to the total ion chromatogram (TIC).

7.2.3 Data analysis

Statistical analyses were performed using the SAS (SAS Institute Inc., 1989) statistical package. A t-test (assuming unequal variance) was carried out to examine differences in total oil content extracted from healthy phloem and the new phloem formed after wounding. An analysis of variance (ANOVA) was performed using the General Linear Models (GLM) procedure, followed by the least significant difference (LSD) test in order to assess the differences of engelitin, catechin, pedunculagin, polymeric PAs, and FPCs concentrations to that found in healthy sapwood, healthy phloem and wound-associated phloem.

Fig. 7.1. Sunken necrotic lesions (arrows) observed on the aerial bark of 3-year-old *E. globulus* naturally infected with *Cytonaema* sp. Bar = 1 cm.

Fig. 7.2. *Cytonaema* sp. isolated from *E. globulus* in Tasmania.

a: Conidiomata (arrow) on bark surface. Bar = 500 μ m

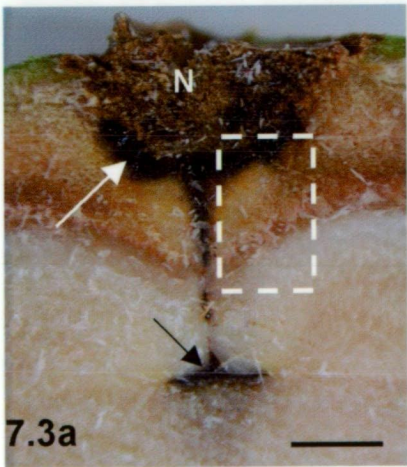
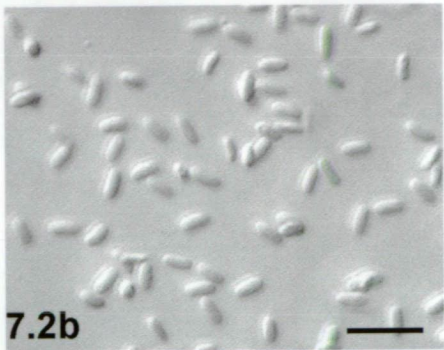
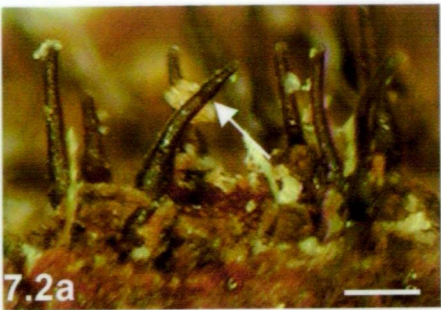
b: micro-conidia. Bar = 5 μ m.

Fig. 7.3. Transverse sections of host bark response to severe canker infection by *Cytonaema* sp.

a: Macrograph of infected bark from a 3-year-old *E. globulus* showing point of cambial dieback (black arrow) and layer of dark extractives (white arrow) produced adjacent to necrotic tissue (N). Bar = 2 mm.

b: Micrograph of section stained with toluidine blue from area highlighted in Fig. 3a. Note the production of traumatic oil glands (*) and wound periderm (arrow). Bar = 200 μ m.

CZ = cambial zone, HP = healthy phloem, X = xylem.



7.3 RESULTS

7.3.1 Anatomical characterization of host response

Observations

Healthy secondary phloem of 3-year-old *E. globulus* consisted of layers of phloem fibers, sieve cells, phloem parenchyma cells (that often contain calcium oxalate crystals or polyphenolic compounds), ray parenchyma and a single periderm, usually parallel to the tree circumference. In addition, oil glands, spherical in shape and of variable sizes, were present in the outer phloem (Fig. 7.4). Necrotic tissue was distinguished from healthy tissue by the presence of brown insoluble deposits in the lumina of parenchyma cells external to the WP (Fig. 7.5a).

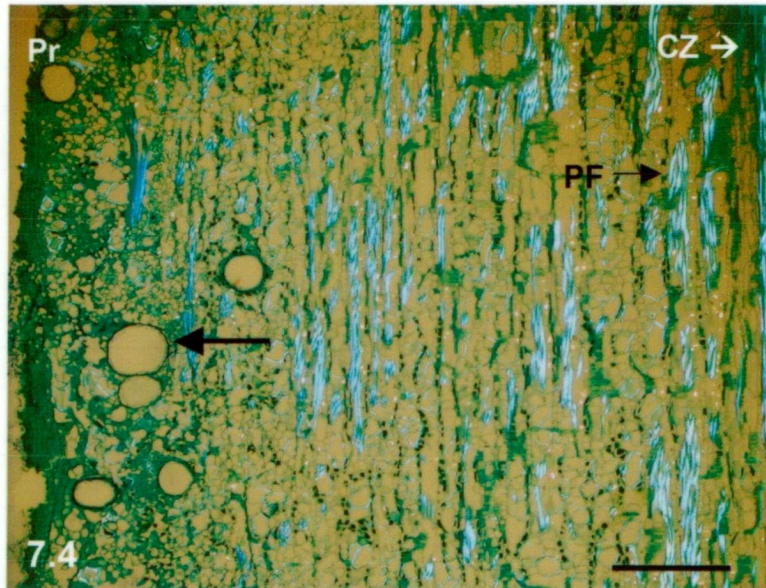


Fig. 7.4. Light micrograph (radial longitudinal) of healthy phloem. Note presence of spherical oil glands (arrow) in the outer phloem. PF= phloem fibres, Pr = periderm; CZ = cambial zone. Stain: toluidine blue. Bar = 250 μ m.

The lesion margin (LM) of the canker-infected bark was morphologically distinct from healthy phloem, its characteristics varying between lesions, depending on severity of canker infection. For example, in superficial infections (in which only the phloem was affected), two different LMs, separating the necrotic region from the underlying healthy tissues were observed (Fig. 7.5). The first LM was characterized by a fully developed WP consisting of a single layer of suberized square thin-walled phellem cells followed by either one to several (up to 11) layers of rectangular thick-walled phellem cells (Fig. 7.5a-c). These thick-walled phellem cells tested positive for the presence of lignin and suberin when treated with phloroglucinol-HCl, and viewed under bright field (Fig. 7.5b) and UV light, respectively (Fig. 7.5c). The cell walls of phloem fibre and many parenchyma cells found externally adjacent to the WP stained positive when treated with phloroglucinol-HCl (Fig. 7.5b). The cell lumens of some phelloderm layers were often infused with polyphenolic material as indicated by their brown colour (Fig. 7.5a).

The second LM type formed around superficial cankers was less organized in structure. A completely differentiated WP similar to that observed with the first LM type was not evident (Fig. 7.5d). Instead, discontinuous zones of meristematic activity in which new cells of varying shape and size had been produced characterized this LM. The cell walls of both extant phloem fibre and phloem parenchyma cells, as well as new parenchyma cells stained strongly for the presence of lignin with phloroglucinol-HCl (Fig. 7.5e). In contrast, the presence of suberin in cells walls was generally most evident in the new parenchyma cells and not in cells extant at the time of infection (Fig. 7.5f).

Figs. 7.5a and 7.5g illustrate what was considered a significant observation for both lesion types, the detection of traumatic parenchyma cells located between the necrotic tissue and the WP, which stained a positive blue-green for the presence of monomeric flavan-3-ols as well as corresponding oligomeric proanthocyanidins as indicated by the DMACA stain. These traumatic parenchyma cells are hereafter referred to as 'induced polyphenolic parenchyma' to differentiate from other parenchyma cells such as those found in tissue

which apparently contained polyphenolic compounds in their cell lumens but did not stain positive under DMACA treatment. In some microscopic examinations, it was possible to observe structural responses at varying stages of anatomical development. From these sections, the induced polyphenolic parenchyma appeared to have been derived prior to the formation of the WP, arising from the hypertrophic dedifferentiation of pre-existing parenchyma cells positioned immediately internal to and abutting the necrotic tissue.

In examples of severe infections in which the vascular cambium had been killed, the formation of a WP was not the only structural response observed to *Cytospora* sp. in the aerial bark. The new phloem formed subsequently also contained irregularly shaped secretory cavities often distributed in clusters within the traumatic parenchyma. These secretory cavities were lined with distinct epithelial cells suggesting these structures to be oil glands (Fig. 7.3b). The cell walls of some mature oil glands stained positive for the presence of Sudan-positive material and autofluoresced under UV light, indicating the presence of suberin-like substances (Appendix 7.2). As similarly observed for superficial cankers, induced polyphenolic parenchyma was also detected at the interface between ligno-suberised boundary zone and necrotic tissue.

In some cases, regardless of severity of infection, mechanical phloem sclereid cells, characterized by thick, lignified polylamellate secondary walls (Quiló *et al.*, 1999) and roughly spherical in shape were often observed within the traumatic parenchyma. In all microscopic examinations, fungal hyphae were only detected on the interior side of the lesion margin and never detected in the healthy tissue produced after the new WP.

Fig. 7.5. Light micrographs (radial longitudinal) showing host response to superficial canker infection by *Cytospora* sp. in 3-year-old *E. globulus*. Outside bark in all figures is orientated toward top of plate.

a-c: A continuous wound periderm ({) comprising of

a: induced polyphenolic parenchyma (as indicated by DMACA stain - arrow) (Note the brown lumina of the phelloderm cells)

b: lignified thin and thick-walled phellem cells (Stain: phloroglucinol-HCl. Viewed under bright field)

c: suberized thin and thick-walled phellem cells. (Stain: phloroglucinol-HCl. Viewed under UV light with a 410-nm barrier filter). Bar in (a-c) = 150 μ m.

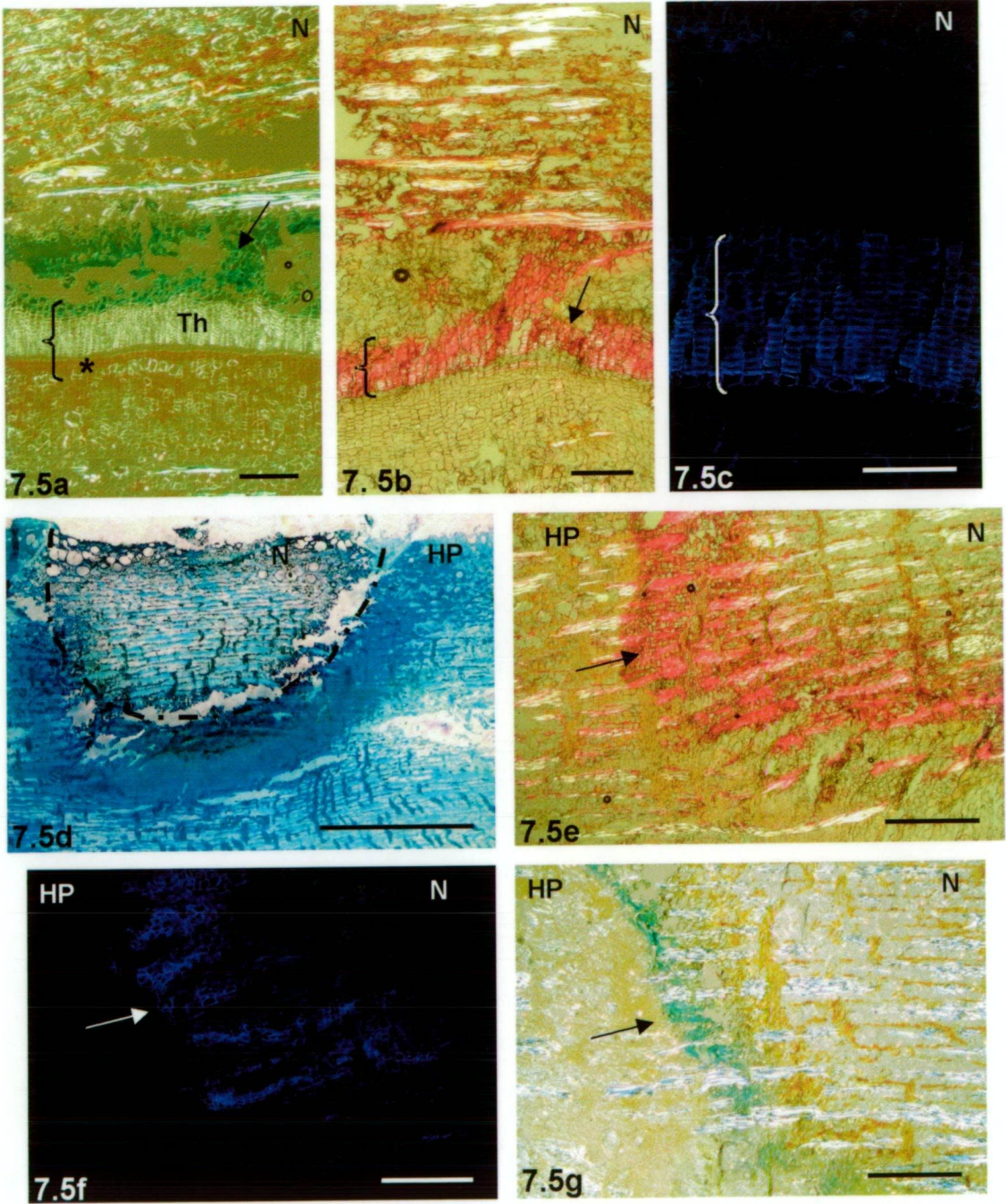
d-g: Section of a typical discontinuous wound periderm at interface of healthy and necrotic phloem (dashed line) (d: Stain: toluidine blue. Bar = 2 mm) comprising of

e: lignified parenchyma cells (Stain: phloroglucinol-HCl. Viewed under bright field)

f: suberized parenchyma cells (Stain: phloroglucinol-HCl. Viewed under UV light with a 410-nm barrier filter). Autofluorescence indicates the presence of suberin in cell walls (white arrow).

g: induced polyphenolic parenchyma. Stain: DMACA. Bar in (e-g) = 300 μ m.

HP = healthy phloem, N = necrotic tissue, Th = thick-walled phellem cells.



7.3.2 Chemical characterization of host response

A complex range of organic compounds including flavonoid glycosides (e.g. engelitin), hydrolysable tannins (eg. pedunculagin), polymeric PAs, FPCs and volatile terpenes (Fig. 7.6) were detected in the lesion margin of infected phloem. These compounds were either not detectable in healthy tissue or only present at significantly lower concentrations than in the lesion margin. More specifically, the lesion margin contained significantly greater concentrations of polymeric PAs (>6 times), FPCs (>4 times), pedunculagin (>15 times) and catechin (>7 times) than either healthy phloem or sapwood. The flavonoid glycoside, engelitin was present in the lesion margin of infected tissue only and not in healthy phloem.

The total oil concentration of terpenes in the new phloem formed subsequently to severe canker formation was significantly higher (> 3 times, $T_6 = 2.45$; $P = 0.012$) than that of healthy phloem (Table 7.1). Fifteen oil components were identified in the extracts (Table 7.2). The main terpenes detected in the lesion margin and in the healthy phloem tissue were aromadendrene, α -pinene, 1,8-cineole, globulol and viridiflorene, in decreasing order of relative abundance (Table 7.2). Significantly greater amounts of α -gurjunene, alloaromadendrene, viridiflorene were present in the lesion margin than healthy phloem. Conversely, significantly lower concentrations of aromadendrene and globulol were detected in the lesion margin compared to healthy phloem.

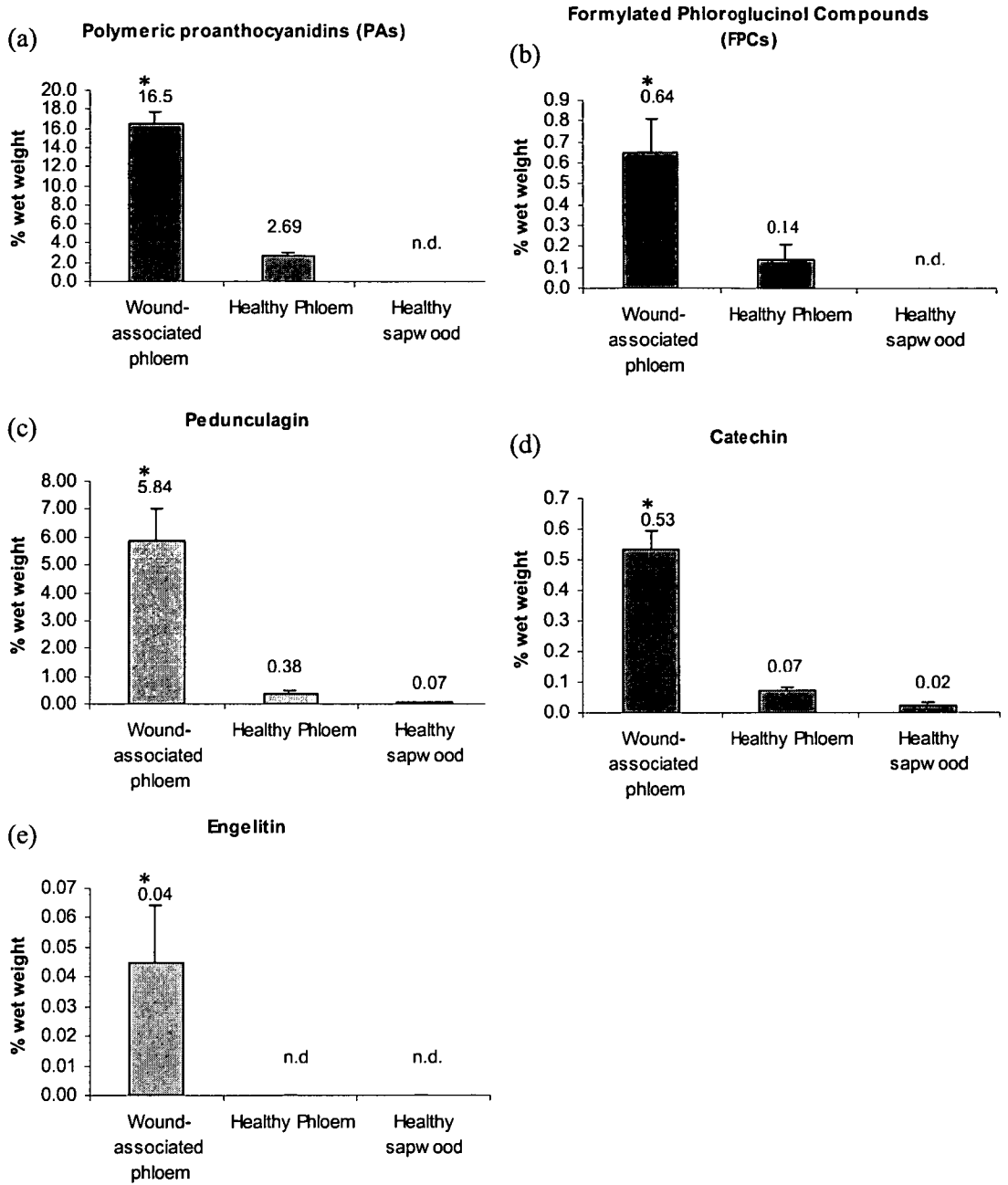


Fig. 7.6. Average concentration (mean \pm SE) of 5 compound classes detected in healthy sapwood, healthy phloem and wound-associated phloem extracts of *E. globulus* naturally infected with *Cytospora* sp. n.d.= not detected. Each column represents the average concentration of 4 replicates. An asterisk indicates significant difference at $P < 0.05$ by protected LSD in ANOVA. (a) $F = 135$, $P < 0.001$; (b) $F = 21.7$, $P < 0.001$; (c) $F = 9.79$, $P < 0.01$; (d) $F = 61.1$, $P < 0.001$; (e) $F = 4.76$, $P < 0.05$.

Table 7.1. Total oil yields in the dichloromethane extracts of 3-year-old *E. globulus* naturally infected with *Cytospora* sp. Mean \pm SE. Sample size is shown in parentheses.

Tissue type	total oil content (wet weight %)
Healthy phloem	0.23 % \pm 0.002 (n = 4)
Wound-associated phloem	0.89 % \pm 0.2 (n = 7)

^A Total oil contents (as % of wet weight) are expressed as 'cineole equivalents' ($T_6 = 2.45$, $P = 0.012$).

Table 7.2. Compounds identified and their relative percentage contribution to the TIC in wound-associated phloem extracts of 3-year-old *E. globulus* naturally infected with *Cytospora* sp. Mean \pm SE

Oil compounds	Tissue type ^A		
	Healthy phloem	Wound-associated phloem	Significance
aromadendrene	26.8 \pm 1.3	22.6 \pm 0.9	*
α -pinene	17 \pm 2.9	17.6 \pm 0.5	ns ^B
1,8-cineole	16.8 \pm 2.1	15.3 \pm 0.7	ns
globulol	10.1 \pm 0.4	8.8 \pm 0.1	**
α -phellandrene	7.5 \pm 2.5	4.0 \pm 2.0	ns
viridiflorene	6.26 \pm 0.6	8.8 \pm 0.3	**
α -gurjunene	3.9 \pm 0.3	5.3 \pm 0.3	*
alloaromadendrene	2.9 \pm 0.1	3.4 \pm 0.1	*
ledol	2.4 \pm 0.2	2.14 \pm 0.1	ns
viridiflorol	1.7 \pm 0.1	1.7 \pm 0.1	ns
terpinyl acetate	1.5 \pm 0.7	0.6 \pm 0.6	ns
limonene	1.4 \pm 0.1	1.7 \pm 0.1	ns
calamenene	1.2 \pm 0.3	1.1 \pm 0.05	ns
Phenol M.W. 252	0.8 \pm 0.3	2.1 \pm 1.3	ns
β -gurjunene	0.7 \pm 0.2	1.2 \pm 0.07	ns

^A Percentage of compounds based on an average of 4 replicates for healthy phloem extracts and 7 replicates for wound-associated phloem extracts. ^B Not significant. * Significant at $P < 0.05$. ** Significant at $P < 0.01$.

7.4 DISCUSSION

Certain host characteristics, including the ability to form various anatomical and structural barriers, and secondary metabolites in response to wounding or pathogen attack have been implicated as important chemical and physical factors contributing to bark resistance of many woody tree species (Mullick, 1977; Woodward and Pearce, 1988a; 1988b; Biggs 1992; Robinson and Morrison 2001; Solla *et al.*, 2002). For the first time, both the chemical and anatomical aspects of host responses of *E. globulus* aerial bark to natural infection by *Cytospora* sp. are presented. As discussed below, the different morphology of the WP, and the detection of induced polyphenolic parenchyma, and traumatic oil glands in the LM suggest that the *E. globulus* LM formed in response to *Cytospora* sp. infection differs from many of the other woody tree species previously studied.

Microscopic examination of infected bark showed that the morphological characteristics of the LM varied. The various LMs observed could be roughly divided into three main categories. Two were associated with superficial cankers and the third, with severe cankers. A common feature for all LMs was the detection of induced polyphenolic parenchyma between necrotic tissue and WP. This finding has not been noted elsewhere in the bark of either eucalypts or other woody tree species. Workers have previously only detected the presence of phenolic-occluded cells in bark tissues extant at the time of wounding (e.g. tanniferous parenchyma in *E. marginata* (Tippett *et al.*, 1983; Tippett and Hill, 1984), diazotized o-toluidine positive parenchyma in *Picea sitchensis* (Woodward 1992), and more recently polyphenolic parenchyma in *Picea abies* (Nagy *et al.*, 2000). Given that the induced polyphenolic parenchyma was observed to form prior to the establishment of a WP, these cells may be part of the initial sequence of wound-induced events involved in restoration of bark microenvironment and possible *de novo* sites of synthesis of certain phenolics. The discovery of induced polyphenolic parenchyma is an important finding and as such deserves further study. The occurrence of parenchyma cells filled with secondary metabolites has previously been considered an important constitutive defence mechanism against pathogens and insects in *Picea abies* (Francheschi *et al.*, 1998; Nagy *et al.*, 2000).

In woody tree species including eucalypts, the formation of a WP has been suggested to be an important resistance factor against a variety of biotic agents, most likely by acting as a structural barrier to further penetration by fungi and allowing restoration of microenvironment (Tippett and Hill, 1984; Ichihara *et al.*, 2000; Robinson and Morrison, 2001). In the present study, the WP formed in response to superficial canker infection in *E. globulus* was anatomically different to that observed for healthy periderm. The WP was characterized by a single layer of suberized thin-walled phellem cells followed by up to 11 layers of very thick lignified and suberized phellem cells. In contrast, the phellem of healthy periderm observed in the present study and that reported for other eucalypt species including *E. globulus*, generally consisted of a single layer of thin-walled suberized cells and only up to three layers of thick-walled phellem cells (Chattaway, 1952; Tippett *et al.*, 1983; Quilho *et al.*, 1999). Given that lignified and suberized tissues are highly resistant to microbial degradation and desiccation (Biggs, 1992), then future studies may find that resistance against certain pathogens in *Eucalyptus* is positively related to the number of these thick-walled phellem layers produced in the LM as similarly suggested in the roots of *Larix occidentalis* against infection by the root rot *Armillaria ostoyae* (Robinson and Morrison, 2001).

New phloem formed in response to severe infection contained traumatic oil glands. Oil glands occur naturally in the plant organs of many *Eucalyptus* species including leaves, root bark, the stem pith, phloem, peduncle and floral buds, and fruit, depending on the species (Carr and Carr, 1969). However, the occurrence of traumatic oil glands in wound-associated phloem has just been recently discovered in eucalypts after mechanical wounding (Chapter 6) and is reported here for the first time following fungal infection. Neither Tippett *et al.*, (1983), or Tippett and Hill (1984), studying the anatomical responses that occurred in the bark of *E. marginata* following inoculation with *Phytophthora cinnamomi* (a highly pathogenic fungus) made any mention of traumatic oil glands forming part of the lesion margin in *E. marginata*. The cell walls of some oil glands were observed to autofluoresce indicating the presence of suberin-like compounds. While this occurrence has been

previously alluded to in studies investigating the developmental anatomy of oil gland formation found in the fruit seeds of various eucalypt species (Carr and Carr, 1970), this is the first study to unequivocally confirm this finding. Other specialized secretory cells such as those resin canals in pines have similarly shown to produce suberized cells walls (Ishida *et al.*, 1993).

Natural infection by *Cytospora* sp. induced important modifications in the bark chemistry of *E. globulus*, as indicated by the histochemical studies and confirmed by GC-MS, HPLC-UV and HPLC-MS analysis. In comparison to either healthy bark or sapwood, canker-infected bark contained significantly greater concentrations of both non-volatile and volatile secondary metabolites of at least five classes of constitutive and induced secondary compounds (including PAs, FPCs, hydrolyzable tannins (pedunculagin), flavonoid glycosides (engletin) and terpenes). Increased concentrations of secondary compounds, particularly phenolics and/or terpenes have been reported to occur in the lesion margins of many conifers and are generally considered to be implicated in protection against pests, diseases and damage (Raffa *et al.*, 1985; Woodward and Pearce, 1988b; Evensen *et al.*, 2000; Viiri *et al.*, 2001). Clearly though, a mere change in the concentration of secondary metabolites is not sufficient evidence to support a direct antimicrobial role against pathogens and bark beetles. While *in vitro* bioassays have demonstrated a direct toxic effect of some secondary metabolites including those formed in the lesion margin of *E. globulus* and other woody species against fungi and bark beetles (Raffa *et al.*, 1985; Delorme and Lieutier, 1990; Evensen *et al.*, 2000), the biological significance of the many other compounds detected in the lesion margin extracts of *E. globulus* remains unclear. We suggest that the range of compounds found in the lesion margin may reflect the possible diverse functions of the secondary metabolites in tree defence and regeneration following wounding as suggested by Schultz and Nicholas (2000). For example, some compounds may be precursors for the phenolic components of the periderm. Other compounds such as high molecular weight PAs and hydrolyzable tannins, which have found to act as strong antioxidants *in vitro* (Hagerman *et al.*, 1998) may scavenge deleterious free radicals generated by wounding (van Doorslaer *et*

al., 1999) and/or by white and brown rot fungi. The free radicals produced by the fungi are thought to have a primary role in the depolymerization of wood cell material (Pearce, 2000).

The anatomical responses to *Cytonaema* sp. in the bark of *E. globulus* appear to differ to those reported for more studied tree species such as conifers and broad-leaved trees. The physical barriers of the WP comprising multiple layers and induced polyphenolic parenchyma in addition to the production of secondary metabolites would appear to be effective in restricting the spread of stem cankers caused by *Cytonaema* sp. Clearly, further inoculation tests will be required to determine the effectiveness of lesion margins in confining the spread of this fungus. Moreover, we suggest that the varying host responses observed in the present study may be due to differences in fungal virulence, seasonal effects or they may relate to variations in lesion age as lesions of different ages were sampled. Therefore to reduce potential sources of variation, future work should study the sequence of events leading to lesion formation following controlled artificial inoculation with fungi of varying virulence at different times of the year. Ultimately, results from such experiments may provide a basis to determine the resistance mechanisms involved against pathogens that cause stem cankers as have been found in other host-pathogen interactions. For example, clones of *Cupressus sempervirens* L. resistant to a cypress canker caused by *Seiridium cardinale* (Wag.) Sutton and Gibson were found to be characterized by a continuous ligno-suberised boundary zone of four to six layers around the lesion margins compared to only two to four layers in susceptible clones (Spanos *et al.*, 1999). The incidence of damage caused by canker causing fungi are likely to become sufficiently severe to warrant their control in eucalypts plantations, particularly as plantations are increasingly managed with intensive practices that result in wounding. Therefore, an improved understanding of the host response at the morphological and chemical level may offer a basis for selection of resistance against pathogenic fungi that cause stem cankers.

APPENDIX 7.1

Preliminary pathogenicity trial of *Cytonaema* isolates on *E. globulus* seedlings

Materials and methods:

In early autumn of 2002, ten pot-grown *E. globulus* seedlings (15 months of age with diameter of ~ 13 mm) were used for the inoculation test. Seedlings were grown outside in a commercial soil mix and subject to regular watering. Using a cork borer, wounds (1 cm diameter) were made at two points along the stem; 10 cm and 30 cm above soil level to the depth of the cambium. For each tree, a 1 cm³ piece of agar from the edge of an actively growing culture (3-5 weeks old) was placed, mycelium surface down into wounds located at the 30 cm inoculation point while sterile agar pieces (controls) were placed into wounds located at the 10 cm inoculation point. Since two colony types, referred to as isolate *Cytonaema* sp. A (Fig. A7.1a) and isolate *Cytonaema* sp. B (Fig. A7.1b) had been isolated from naturally infected bark; the seedlings were divided to allow 5 replicates for each culture. All wounds were sealed with Parafilm to prevent desiccation and cross-contamination of inoculation points. 40 days after inoculation, responses including the length of any necrotic lesions and/or fruiting bodies to inoculation were recorded.

Results:

Observations; Artificial inoculations with *Cytonaema* sp. A did not result in any necrotic lesion along the stem of *E. globulus* seedlings (Fig A7.1c). In contrast, *Cytonaema* sp. B was shown to successfully induce necrotic lesions following artificial inoculations. In all cases, necrotic lesions were greater than 1 cm in length (Fig. A7.1d). Raised black fruiting bodies could be seen upon closer inspection of the necrotic lesion (Fig. A7.1e).

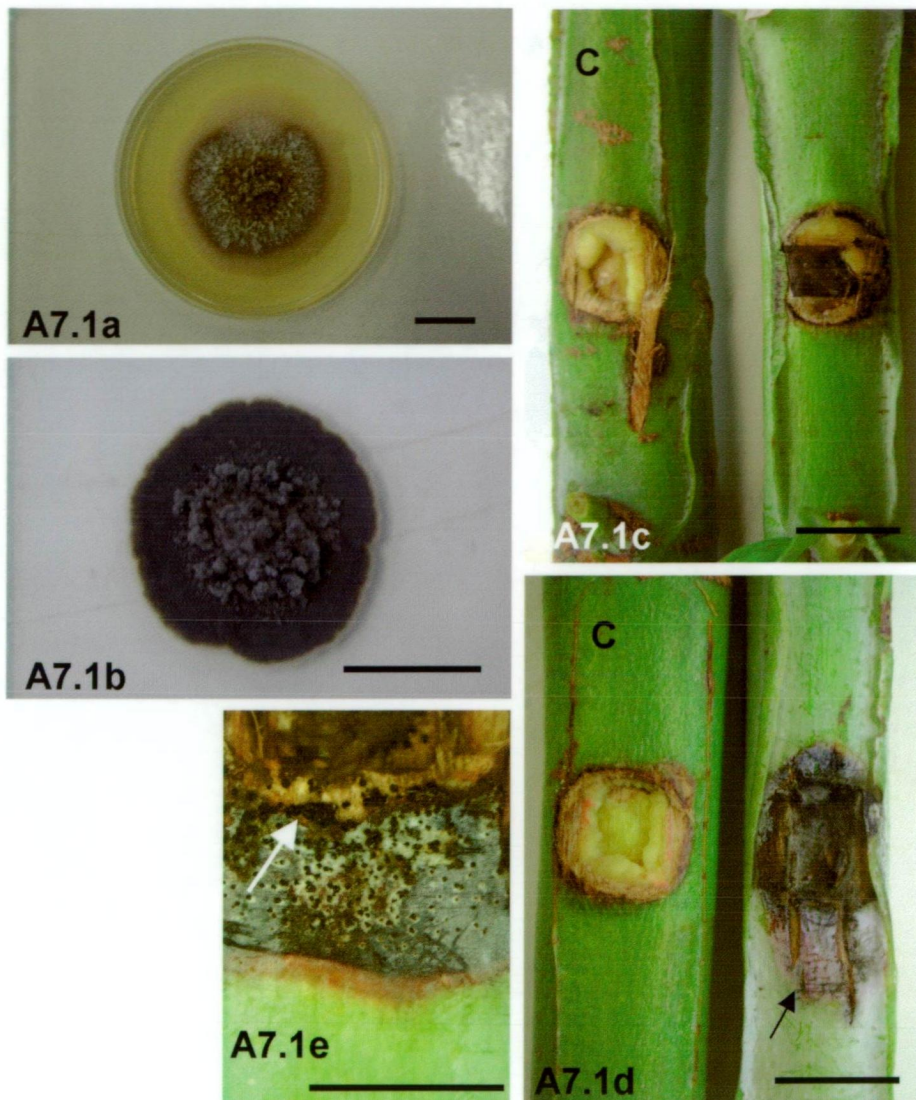


Fig. A7.1a. Pure culture of *Cytonaema* sp. A grown on MEA. 7.1b Pure culture of *Cytonaema* sp. B grown on MEA. Bar ~ 2 cm. Host response of *E. globulus* seedlings inoculated with cultures of *Cytonaema* A (A7.1c) and B (A7.1d). Note induction of necrotic lesion (arrow) and presence of raised black fruiting bodies (arrow) after inoculation with *Cytonaema* sp. B. (A7.1e). C = control. Bar ~ 1 cm.

APPENDIX 7.2

Detection of suberin-like material in the cell walls of traumatic oil glands as indicated by autofluorescence and Sudan Black histochemical stain.

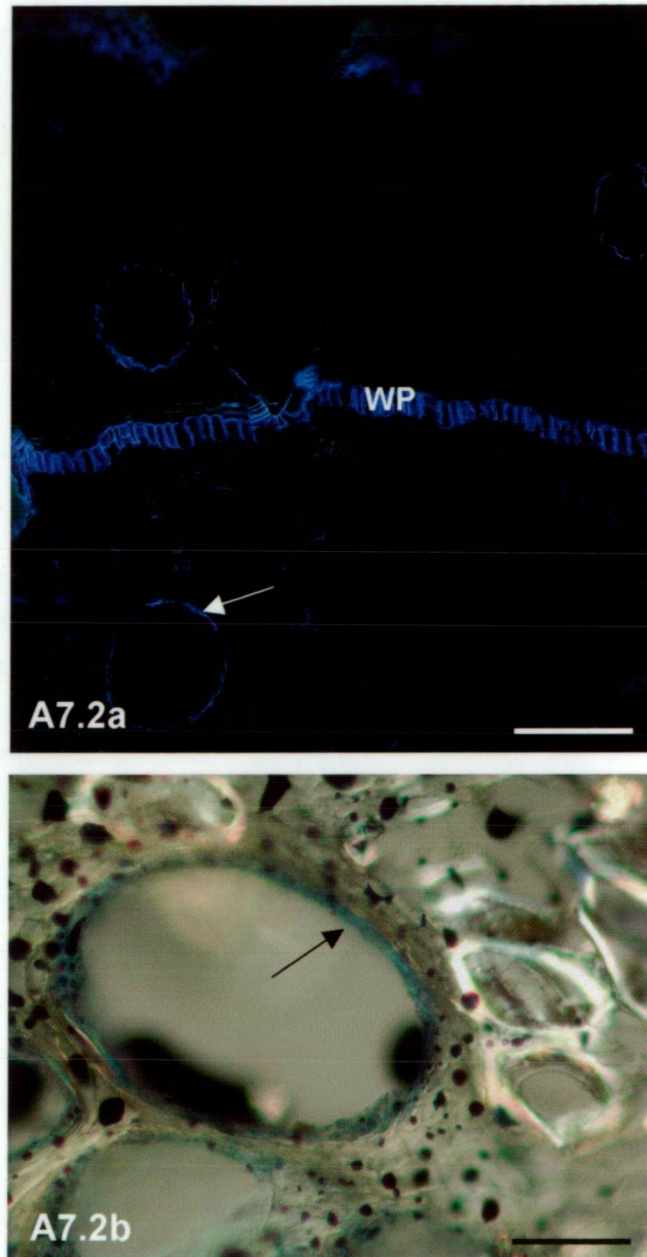


Fig. A7.2. Transverse section showing suberin-like compounds detected in the cell walls of traumatic oil glands in *E. globulus*. A7.2a: Stained with phloroglucinol-HCl and viewed under UV light with 410 nm barrier filter. Autofluorescence indicates presence of suberin in cell walls (arrow). Bar = 100 μm . A7.2b: Stained with Sudan Black. Note positive colour change indicates presence of suberin (arrow). Bar = 30 μm . WP = wound periderm.

Chapter 8

Antioxidant activity of *Eucalyptus globulus* wound wood extractives

Abstract

Antioxidant activity of crude *Eucalyptus globulus* extracts obtained from new wound wood was measured using the WST-1 assay. This *in vitro* assay determined the activity of superoxide radicals scavenging in a xanthine oxidase system. The 50% inhibition (IC₅₀) of formazan formation was estimated for fractionated samples and purified compounds isolated from the crude wound wood extract i.e. engelitin and pedunculagin. Standards of gallic acid and pentagalloyl-glucose were also measured. Fraction II as well as the compounds pentagalloyl-glucose and pedunculagin showed the highest levels of antioxidant activity. Subsequent analysis of Fraction II by HPLC coupled to negative ion electrospray mass spectrometry revealed the fraction to be dominated by hydrolysable tannins including pedunculagin, di-, tri- and tetragalloyl-glucoses. These preliminary results provide some evidence for an antioxidant role of secondary metabolites in tree wound repair and defence.

8.1 INTRODUCTION

Plants produce an array of antioxidant compounds and enzymes including ascorbate, glutathione, carotenoids and superoxide dismutases to scavenge the free radicals and other oxidative species generated in response to both biotic and abiotic stress conditions (Grace and Logan 2000). There is now increasing evidence that a range of polyphenolic compounds, also have free-radical scavenging activity i.e. the ability to quench various oxidizing free radicals, including the superoxide and hydroxyl radicals that damage DNA, proteins and membrane lipids (Jansen *et al.*, 1998; Wood *et al.*, 2002). More specifically, both proanthocyanidins and hydrolysable tannins have been shown to act as strong antioxidants *in vitro* (Rice-Evans *et al.*, 1996; Hagerman *et al.*, 1998). Proanthocyanidins are oligomeric and polymeric flavonoid compounds composed of flavan-3-ol subunits whereas hydrolysable tannins include both ellagitannins (esters of hexahydroxydiphenoyl groups with a sugar core and often galloyl groups) and gallotannins (a sugar core substituted only with galloyl groups) (Hagerman and Butler 1991).

Recently, *Eucalyptus globulus* (Labill.) has been shown to produce a wide range of polyphenolic compounds in new wound wood. More specifically, a variety of hydrolyzable tannins, proanthocyanidins, flavonone glycoside, stilbenes, formylated phloroglucinol compounds (FPCs) as well as terpenoids were detected. In contrast, these compounds were either undetectable in healthy sapwood or present at only trace levels (Chapters 4 and 5). In other woody tree species, the induction and accumulation of secondary metabolites in the living sapwood have long been implicated as playing an antimicrobial role (see detailed reviews by Pearce 1996 and Yamada 2001). Although *in vitro* bioassays using crude extracts obtained from wound wood demonstrated clear antimicrobial activity against decay fungi and gram-positive bacteria, studies also showed that the production of secondary metabolites appeared largely independent of the occurrence of fungal colonization (Chapters 3 and 4). In these studies, we proposed that the diverse range of metabolites induced in the wound wood might have multiple roles, particularly when considering the complexity of secondary metabolites detected in eucalypt wound wood as compared to healthy sapwood. Given that deleterious free radicals are generated from wounding (van Doorslaer *et al.*, 1999) and/or infection by white and brown rot fungi (Hirano *et al.*, 1997), we suggested that the secondary metabolites may have an antioxidant role.

In an attempt to determine whether or not the secondary metabolites found in the wound wood of *E. globulus* have an antioxidant role, we tested the antioxidant activity of various fractions and compounds using a recently developed assay involving a xanthine oxidase system and the water soluble tetrazolium salt, (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzene disulfonate sodium salt) (WST-1) (Ukeda *et al.*, (1999) and Peskin and Winterbourn (2000)).

8.2 MATERIALS AND METHODS

Crude extracts of *E. globulus* wound wood in 70 % aqueous acetone were obtained from previous drill wounding experiments (Chapters 4 and 5) and were pooled together to obtain a

total of 1 g of freeze dried extractives (stored at 4 °C) for fractionation and subsequent antioxidant assays.

8.2.1 Fractionation of *Eucalyptus globulus* wound wood extracts (see Fig. 8.1)

Prior to chromatographic fractionation, the crude sample was extracted with hexane to remove non-polar compounds such as FPCs. The remaining insoluble fraction (0.95 g) was fractionated by chromatography on Sephadex LH-20 with elution by ethanol, methanol and 70 % aqueous acetone, successively to collect a total of 8 fractions. One of the methanol fractions, referred to as Fraction I, was further fractionated by preparative high performance liquid chromatography (HPLC) with ultraviolet detection to obtain the engelitin and polyphenol fraction. HPLC analyses were carried out on a HPLC pump 880-PU (JASCO Co., Ltd., Japan), with detector wavelength at 280 nm (UV/VIS Detector 870-UV, JASCO Co., Ltd., Japan). The column was a Develosil ODS -10 C₁₈ column (20 mm i.d. x 250 mm, Nomura Chemical, Ltd., Japan). The solvent system was a 1:1 solution of methanol: 0.01% aqueous trifluoro acetic acid with a flow rate of 10 mL min⁻¹ at room temperature. Using the same HPLC system, one of the 70 % aqueous acetone fractions was further fractionated to obtain pedunculagin and Fraction II. The polyphenol fraction was further purified by column chromatography on Sephadex LH-20 with elution by ethanol, methanol and 70 % aqueous acetone, successively. The methanol fraction was further purified by column chromatography on Toyopearl HW-40F with ethanol, methanol and 70 % aqueous acetone, successively. The resultant 70% aqueous acetone fraction was referred to as Fraction III.

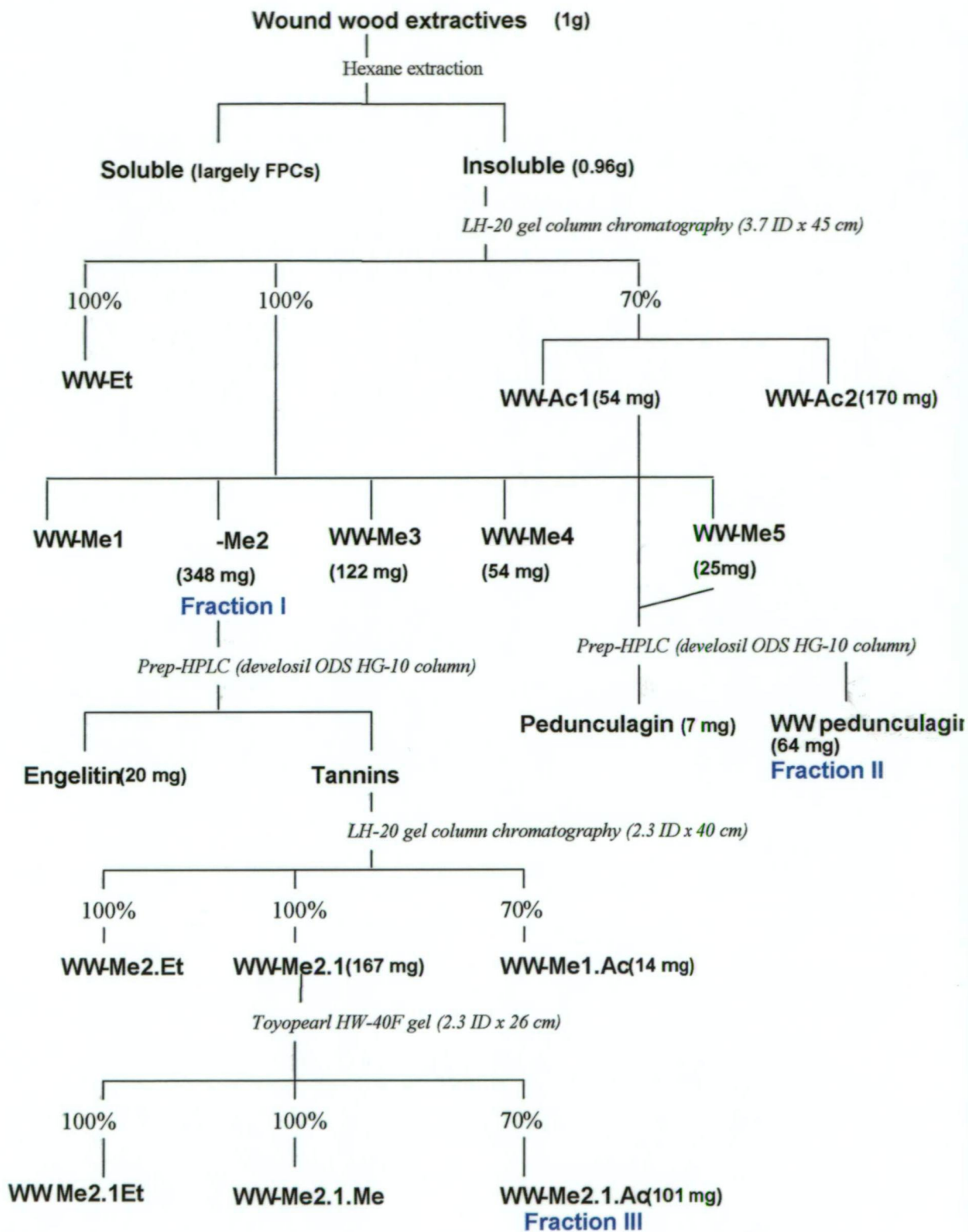


Fig. 8.1. Fractionation scheme of *E. globulus* wound wood (WW) extracts

Note: ID = inside diameter. Fractions chosen for antioxidant assay are written in blue.

8.2.3 WST-1 method for measurement of antioxidant activity

The ability of pedunculagin, engelitin and Fractions I, II and III to scavenge superoxide radicals was measured based on the DOJINDO SOD assay kit-WST (DOJINDO Co., Ltd., Japan). The principal of the WST-1 assay is shown in Fig. 8.2. In the absence of compounds that quench the superoxide anion, the maximum amount of WST-1, is observed in the form of a highly water-soluble formazan dye, clearly detectable by a spectrophotometer. Conversely, in the presence of compound/s that scavenge the superoxide anion, less formazan dye will be produced, the amount providing an estimate of the strength of the compound's antioxidant activity. The standards, pentagalloyl-glucose and gallic acid were chosen as positive controls as they had been identified previously in wound wood extracts (Chapter 4).

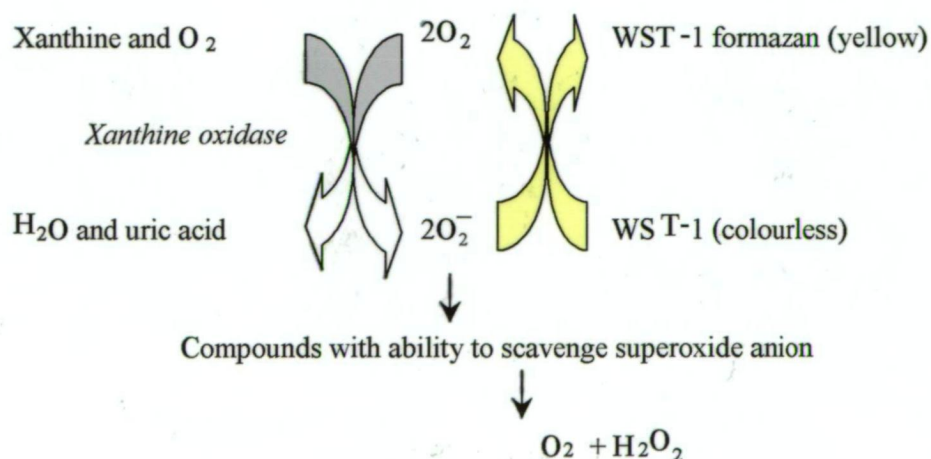


Fig. 8.2. Principle of the antioxidant assay based on the reduction of WST-1

Reagents

WST-1 mixture: WST-1: Xanthine in a 1:9 ratio WST-1 (0.75 mM in 0.1 M HEPES buffer

– Xanthine (0.1 mg mL⁻¹ of xanthine in 0.1 M HEPES buffer (pH 8.3). Stored at 4 °C.

Enzyme solution: 0.5 ml of xanthine oxidase (5.5 units/ml) in 50 ml of 0.05 M sodium phosphate buffer and 0.01 M EDTA (pH 7.5). Stored at 4 °C.

HEPES buffer: 0.1 M HEPES buffer (pH 8.3). Stored at 4 °C.

Procedure

Pedunculagin, engelitin, gallic acid, pentagalloyl-glucose, Fractions I, II and III (1, 0.5, 0.25 and 0.1 mg mL⁻¹ of each sample, respectively) were dissolved in 50 % aqueous dimethyl sulfoxide. To two sets of 50 µl of each sample, 500 µl of WST-1 mixture was added, mixed with a vortex, covered and pre-incubated for 5 min at 37 °C. To the first set of samples, 500 µl of enzyme solution was added, mixed well, covered and then incubated for a further 20 min at 37 °C. To the second set of samples (acting as controls - Blank 2), 500 µl of HEPES buffer was added, mixed and then incubated with a lid for a further 20 min at 37 °C. To ensure standard incubation timing of each sample, the last reagent was added at one-minute intervals. The absorbance of the yellow colour that developed was measured at 450 nm on a Jasco V-520 UV/Vis Spectrophotometer (JASCO Co., Ltd., Japan). In order to obtain 50% inhibition levels (IC₅₀), this procedure was repeated for all samples at 1, 0.5, 0.25 and 0.1 mg mL⁻¹. The data obtained at the 4 concentrations were fit to a line (where scavenging activity was a function of concentration) and the concentration required to inhibit by 50% was calculated from the regression. Calculations of antioxidant activity were obtained using the following equation: [(blank 1- blank 3)-(sample - blank 2)] / (blank 1- blank3) X 100%].

8.2.2 Chemical analysis of Fraction I, II and III by LC-MS

Since the chemical composition of the original wound wood extractives had already been examined (Chapters 4 and 5), chemical analyses were restricted to Fractions I, II and III. Injections of 5 µL were analysed using HPLC-MS. Detailed description of the operating conditions are given by chapters 4 and 5. The identification of hydrolysable tannins was confirmed by comparing their negative ion electrospray ionisation-mass spectrometry (ESI-MS) characteristics with those described previously by Barry *et al.*, (2001) and Davies and Barry (2001), which included the use of several authentic standards. The identification of proanthocyanidins was based on tandem mass spectral data, including data obtained from a previous depolymerisation experiment (Chapter 4).

8.3 RESULTS

8.3.1 Antioxidant activity

With the exception of engelitin, all three *E. globulus* wound wood fractions as well as the original wound wood extractives exhibited antioxidant activity as measured by the WST-1 method (Table 8.1). The highest level of antioxidant activity was observed in the standard penta-galloyl glucose. Out of the three fractions tested, Fraction II had the greatest superoxide radical scavenging activity.

Table 8.1. Antioxidant activity (IC₅₀ : 50 % inhibition levels for each sample (µg/ml)) for different compounds and fractions isolated from *E. globulus* wound wood extracts.

Compound/fraction	IC ₅₀	% inhibition (11.9 µg/mL)
original wound wood extractives	5.6	76
Fraction I	7.2	70
Fraction II	4.7	81
Fraction III	13.0	48
penta-galloyl-glucose *	<4.7	90 (<5 µM)
pedunculagin	5.6	82 (7.3 µM)
gallic acid *	6.0	97 (35.3 µM)
engelitin	negligible activity	0.5

* standard (detected in original crude wound wood extract)

8.3.2.Chemical analysis

Analysis of the three fractions by HPLC-MS showed that each fraction was of a different chemical composition. In order to gain an overview of the major compounds present in each fraction, a summed spectrum was determined between molecular ion (*m/z*) 125 and 1500 (Fig. 8.3). In Fraction I, a wide range of proanthocyanidins were detected including monomers, dimers, trimers and tetramers comprised of both procyanidin and propelargonidin subunits, largely detected as their respective rhamnosides (Fig. 8.3a). In contrast, Fraction II was dominated by the presence of a variety of hydrolysable tannins including ellagic acid as

well as di-, tri- and tetra-galloyl glucoses (Fig. 8.3b). Fraction III was observed as a complex range of unknown higher molecular weight compounds (chromatogram not shown). No hydrolysable tannins were detected in Fraction III and only a few and insignificant amounts of well known condensed tannins were present.

8.4 DISCUSSION

Our results provide some preliminary evidence to suggest that certain polyphenolic compounds (particularly hydrolysable tannins) induced in the wound wood may act as antioxidants, induced to scavenge the free radicals produced during wounding and by decay fungi. More specifically, the original extractives obtained from the wound wood of *E. globulus*, comprising a complex array of polyphenolic and terpenoid-based compounds, were shown to have strong antioxidant activity in the WST-1 assay. Furthermore, Fraction II, revealed to be largely composed of various hydrolysable tannins, exhibited the strongest level of antioxidant activity of the three fractions examined. Additional tests using the purified hydrolysable tannins pedunculagin and penta-galloyl glucose also exhibited similar levels of antioxidant activity to Fraction II.

In the only detailed study of the chemistry of eucalypt sapwood in response to wounding and infection, the detection of pedunculagin (an ellagitannin) at elevated concentrations was always observed in the reaction zone of *E. nitens* in response to fungal inoculation (Barry *et al.*, 2001). Subsequent *in vitro* antifungal bioassays with pedunculagin did not provide evidence to suggest a direct antimicrobial role (Barry 2001). Therefore, the induction of pedunculagin in the compromised sapwood may be primarily for antioxidant function. The potential physiological role of polyphenolic compounds having an antioxidant function has been similarly hypothesised for leaves of the same eucalypt species (Close and McArthur 2002).

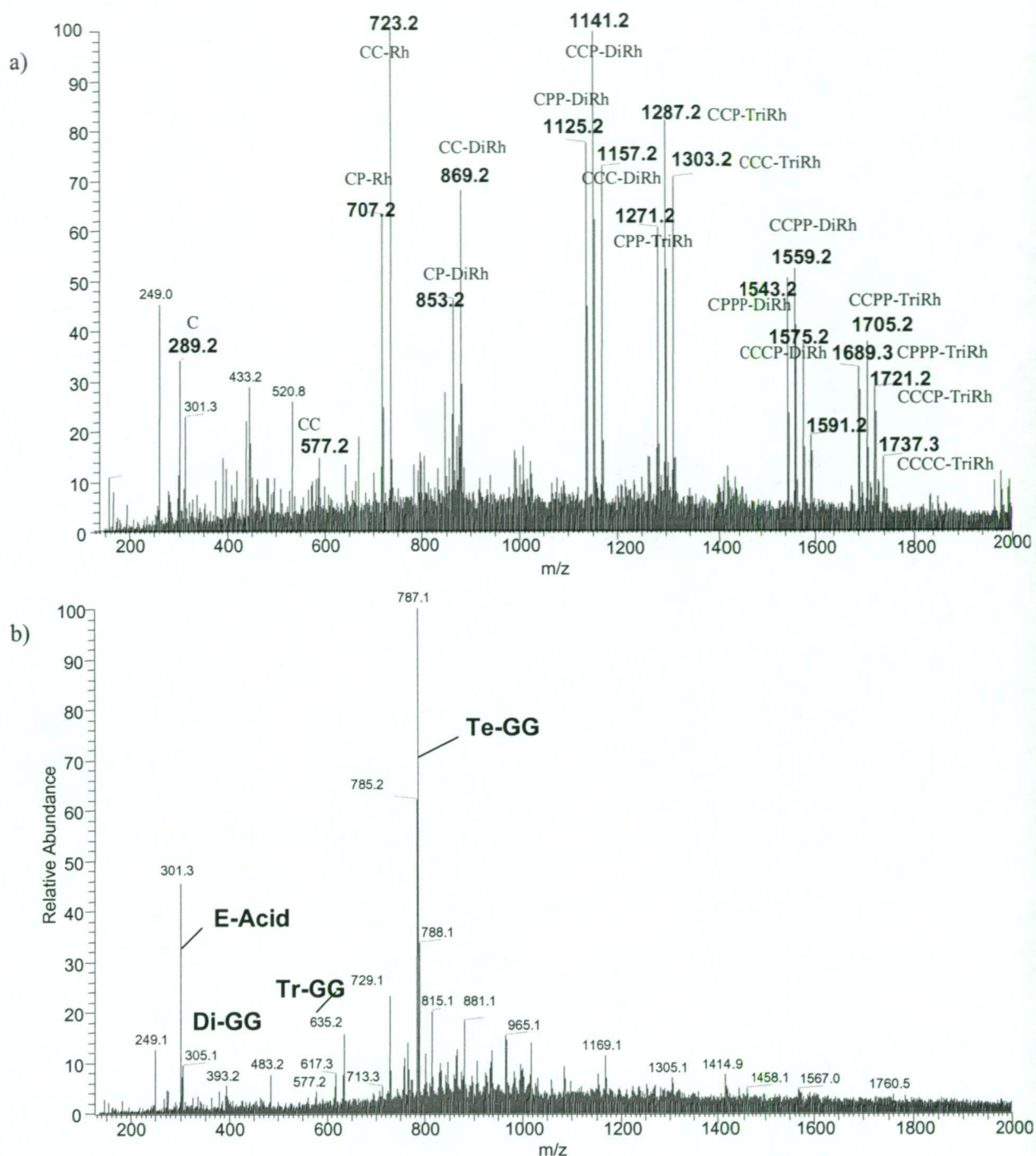


Fig. 8.3. The summed mass spectra in the range of m/z 125-1500 for Fractions I (a) and II (b) obtained from *E. globulus* wound wood by fractionation with column chromatography. Di-GG, Tr-GG, Te-GG = di-, tri-, tetra-galloyl glucoses, respectively; E-Acid = ellagic acid. All numbers in bold are proanthocyanidins. In (a), the putative assignments of proanthocyanidin oligomer type based on detailed tandem mass spectrometry studies are shown. C = catechin or epicatechin unit, P = propelargonidin unit, Rh = rhamnoside unit. Note that no assignment of order of proanthocyanidin subunits is intended; rather, only the proposed degree of oligomerisation and rhamnose substitution is shown e.g. 'CCP-DiRh' implies a trimer with two catechin/epicatechin units and one propelargonidin unit, with two rhamnose units attached.

For example, in a recent study examining temporal variations of tannins, flavonols and anthocyanins in the leaves of *E. nitens* seedlings in response to nutrient treatments, the two-fold higher accumulation of di-, tri-, tetra-, and pentagalloyl glucoses detected in sun-adapted leaves compared to shade-adapted leaves was interpreted as a response to minimising photodamage (Close *et al.*, 2001). Furthermore, a number of flavonoids and tannins isolated from *Eucalyptus* leaves have been reported to show antioxidant properties from *in vitro* assays (Okamura *et al.*, 1993).

The role of secondary metabolites as antioxidants in tree wound repair and defence requires further investigation. This study focused mainly on hydrolysable tannins as potential contributors to antioxidant activity although proanthocyanidins were also shown to have strong antioxidant activity. Given that antioxidant activity has been shown to depend on the number of hydroxyl groups and/or degree of polymerisation (Hodnick *et al.*, 1988), the relatively high levels of antioxidant activity observed for the hydrolysable tannins was not unexpected. Hydrolysable tannins are characterized by the presence of many phenolic hydroxyl groups eg. di-galloyl glucose and pentagalloyl glucose contain six and fifteen groups, respectively (see Barry *et al.*, 2001 for chemical structures of pedunculagin, and di-, tri-, tetra- and penta-galloyl glucose). Interestingly, the compound engelitin, a flavonoid glycoside with a total of three phenolic hydroxyl groups, demonstrated little antioxidant activity. It would seem that antioxidant activity is dependent not just on the degree of hydroxylation but also the hydroxylation pattern on the rings of the flavonoid as summarized by Wood *et al.*, (2002) and Amic *et al.*, (2003). As found in other studies (eg. Hagerman and Butler 1991), the purification of proanthocyanidins to obtain relevant standards for bioassay study is a laborious task. However, with the development of improved fractionation techniques and a better knowledge of the chemistry of wound wood extractives, it may be possible to determine what other polyphenolic compounds induced in wound wood are responsible for antioxidant activity.

CHAPTER 9

Importance of wound wood overlooked in tree

compartmentalization? A case study with *Eucalyptus globulus* and *E. nitens*

Abstract

Trees respond to wounding and infection by 'compartmentalizing' the wounded area to limit the spread of dysfunction and infection in the xylem. Tree compartmentalization involves changes in the biochemistry and morphology of the xylem present at wounding as well as the type of xylem formed after wounding. We have been studying the new tissue formed immediately adjacent to the wound site, which we refer to as wound wood. The vital roles of wound wood in wound closures, re-establishment of the vascular cambium continuity and restriction of gaseous exchange within the dysfunctional xylem have been well studied. In contrast, the ability of wound wood to resist microbial colonisation has received consideration in neither hardwoods or softwoods. Based on our findings from recent chemical and morphological studies of wound responses in two commercially important hardwood species, *Eucalyptus globulus* (Labill) and *E. nitens* (Maiden), we propose a reassessment of the importance of wound wood in tree compartmentalization.

9.1 INTRODUCTION

Despite being the second most popular genus selected for tree plantation in the world (Anon, 2001), the wound responses of *Eucalyptus* spp. have been poorly studied. In an attempt to fill the current lack of knowledge, we investigated both the anatomical and chemical responses to different wounding treatments of two economically important *Eucalyptus* species grown in southern Australia (*E. globulus* and *E. nitens*). An important feature that was consistently observed as forming part of the wound response included the production of dark extractives in the new tissue formed immediately adjacent to the wound site (Fig. 9.1b). Subsequent chemical analysis of these dark extractives revealed the presence of a diverse range of secondary metabolites including polyphenols (eg. hydrolyzable tannins, proanthocyanidins, flavonone glycosides and formylated phloroglucinol compounds) and terpenoids (Chapters 4 and 5) that were either undetectable in healthy tissue or present at only trace levels. To date, this new tissue found adjacent to the wound site, which we refer to as wound wood (WW), has previously never been studied in any eucalypt species.

9.2 TREE COMPARTMENTALIZATION

It is widely accepted that trees respond to wounding and infection by the production of anatomical and biochemical barriers to compartmentalize the spread of dysfunction, infection and decay in xylem. Models explaining the resistance mechanisms observed in limiting the spread of wood discolouration and decay include static reaction zones (Shain, 1967, 1971, 1979), microenvironmental restriction (Boddy and Rayner, 1983; Rayner and Boddy, 1988; Boddy, 1992) and the compartmentalization of decay in trees (CODIT) (Shigo and Marx, 1977; Shigo 1984). The contributions of the various models to explain the observed patterns of tree responses have already been reviewed elsewhere by Pearce, (1996, 2000), Schwarze *et al.*, (1999) and more recently by Yamada (2001). Given that the Modified CODIT model is the only tree defence model that briefly mentions WW (as callus), we shall confine our discussions mostly to the Modified CODIT model, which coincidentally has had the widest impact on our current interpretation of host response to wounding and infection. While, the full scientific merit of this model is still debatable, the model undoubtedly did provide a workable explanation of the decay patterns visually evident in the wood of living trees to the majority of arborists and foresters.

First developed by Shigo and Marx (1977) and then later modified by Liese and Dujesiefken (1988), the Modified CODIT model relates to injuries (e.g. stem wounds) which occur from the outside inwards and which damage the cambium. As illustrated in Figure 9.1, this model attempts to explain how trees respond to wounding by 'compartmentalizing' the wounded area to limit the spread of dysfunction and infection. Four 'walls' that represent both inherent features of the wood, and induced anatomical and chemical mechanisms are proposed to achieve this compartmentalization. Briefly, axial spread of colonising microorganisms restricted by wall 1 represented by the formation of vessel occlusions e.g. tylosis, while radial movement is limited by the often thick-walled and more lignified cells of the latewood (wall 2). Decay spreading in the tangential direction is impeded by activities of the xylem rays e.g. production of defence substances (wall 3). In general, the first three walls are collectively referred to as the 'reaction zone,' whereas the new tissue laid *de novo* by the

vascular cambium in the vicinity of the wounds is known as the 'barrier zone' (wall 4). This wall separates necrotic discoloured xylem formed at the time of injury against the wood formed after injury.

Alternatively, these 'four walls' may represent passive processes rather than active host defence responses directed specifically against microbial invasion as proposed by Boddy and Rayner, (1983) in their microenvironmental restriction model. According to this model, functional sapwood is an inhospitable microenvironment for most fungi. The spread of decay in living sapwood is restricted by the maintenance of hydraulic integrity so that the high moisture content and concomitant low O_2 tension arrest fungal development. Findings from subsequent studies since the publication of these models have shown that the apparent different interpretations of the function of these 'walls' as either active defences against decay or passive repair processes are not so diametrically opposed.

"The proposed active and passive defences are in no way mutually incompatible and elements of all may function in concert to protect the living tree,' as so eloquently suggested by Pearce (1991, 2000). In the Modified CODIT model, the D (which previously had denoted 'decay') has now been replaced to represent 'dysfunction' (damage) or 'dessication' (drying out) to account for both the active defence responses and of passive microenvironmental factors involved in tree defence and wound repair (Liese and Dujesiefken, 1988; Schwarze *et al.*, 1999).

9.3 DO *EUCALYPTUS GLOBULUS* AND *E. NITENS* CONFORM TO THE MODIFIED CODIT MODEL?

In eucalypts, the development of decay in stems following wounding appears to be consistent with the spatial patterns typical of most tree species following stem wounding. More specifically, the development of discolouration and decay extends much faster longitudinally than radially or tangentially (Mireku and Wilkes, 1989; White and Kile, 1993). One anatomical feature that is unique to eucalypts is the formation of kino veins in

some eucalypt species. Kino veins are macroscopically visible barrier zones, consisting of a tangential series of radial and transverse parenchyma bridges that inter-connect cavities filled with the polyphenolic deposit known as kino (Wilkes, 1986; Tippet, 1986; Chapters 2 and 3). In other eucalypt species, the barrier zones are anatomically more similar to other trees i.e. characterized by the abundant presence of undifferentiated parenchyma cells that often stained positive for the presence of polyphenolic compounds (Fig. 9.1a) (Wilkes, 1986; Chapter 2). From gross morphological comparisons of eucalypt WW with that observed for other woody angiosperms, the presence of visually obvious dark extractives in the WW (Fig. 9.1b) would appear to be another distinguishing feature of eucalypts. For example, no such extractives were reported to occur in the new tissue formed directly adjacent to the wounding site in black walnut after drill wounding (Smith, 1978; Shigo, 1979). It is important to note that there exist clear anatomical and chemical differences between the dark extractives produced in specialised kino veins and those in the WW as already discussed (Chapter 3).

The anatomical development of WW has only been described for a few woody species eg. *Acer* (Bauch *et al.*, 1980) and *Juglans* (Smith, 1978). In these cases, WW was referred to by a variety of names including callus, callus pad, wound-associated wood, xylem-callus curls and woundwood (Smith, 1978; Bauch *et al.*, 1980; Tippet *et al.*, 1985; Lowerts *et al.*, 1986; Torelli *et al.*, 1994; Smith and Sutherland, 1999). From anatomical studies, the WW of both *E. globulus* and *E. nitens* was found to be highly variable in composition comprising callus and altered wood of increased parenchyma content (Chapter 4). Furthermore, the WW was shown to develop from the proliferation of living parenchyma adjacent to the dead region of the wounding site with the resulting WW eventually growing over the exposed wound, as similarly described by other workers for other tree species (Kuroda and Shimaji, 1984; Tippet, 1986; Kozłowski and Pallardy, 1997; Fink 1999; Oven and Torelli, 1999).

In response to the popularity of the Modified CODIT model, we believe that much of the subsequent work on tree defence has been predominantly directed towards investigating the role of both reaction and barrier zone formation as a mechanism of disease resistance. The

brief mention given in the CODIT model seems inadequate in relation to the vital functions of the WW, that include three well known roles of the wound wood ie. a) assisting with wound closure, b) re-establishing continuity of the vascular cambium and c) restricting gaseous exchange within the compromised xylem. Based on our recent findings in *E. globulus* and *E. nitens*, we suggest an additional role for WW formation; restricting the spread of decay-causing fungi into subsequent healthy sapwood. It is important to note that we are not disputing the significance of BZ formation in tree defence. Rather, we argue that the WW should be given similar consideration as given to the BZ in the Modified CODIT model as discussed below.

9.4 RESTRICT FUNGI INTO SUBSEQUENT HEALTHY SAPWOOD

There is no doubt that the BZ does restrict infection into subsequent tissue. However, given that the very definition of BZ is ‘formation of protective tissue laid down *de novo* by the vascular cambium at the time of wounding as a response to infection and/or injury,’ then the restriction of infection outwards into xylem formed subsequently can only apply to those **tissues located along the plane of the vascular cambium** (Fig. 9.1b). According to Shigo (1984), the barrier zone is functionally the most important barrier, protecting the wood formed after wounding and the cambial tissue. However, one of the key questions raised by the current research must be, what is stopping the spread of fungi from the wound site into the new tissue formed following wounding? Our findings from recent studies suggest that wound wood formation plays a key role in restricting fungal spread into subsequent healthy sapwood. Surely the wound site is also an important source of latent inoculum and therefore, a most vulnerable site in the tree compartmentalization process. From our field studies, if fungal decay was present, it was clear that the infection had not spread into subsequent tissue formed after wounding (Chapters 3 and 4). Moreover, crude WW extracts were shown to possess strong antimicrobial activity in preliminary *in vitro* bioassays against decay fungi and gram-positive bacteria (Chapter 5). The complex array of secondary compounds that were induced in the WW of *E. globulus* and *E. nitens* and not in healthy sapwood, have

generally been implicated in protection against pests, diseases and damage in other tree species (Woodward, 1992; Pearce, 1996; Yamada, 1999).

9.5 IMPORTANCE OF WOUND WOOD VARIES BETWEEN SPECIES?

Variation in rate of wound closure clearly exists between tree species as well as within species. For example, in Norway spruce, *Picea abies*, a 10cm diameter wound is reported to take 25-50 years to close while in eucalypts, only 7-8 years (Vasiliauskas, 2001). In our studies examining wound responses in *E. globulus* and *E. nitens*, wounds created by a 1cm drill treatment was found to occlude within 6 months of wounding while in those created by pruning, closure was complete within 2 years (Chapters 3 and 6). This marked difference in rate of wound closure between the more studied *Pinus* species and the less well studied eucalypts may provide a possible reason for the lack of studies on WW in the past. The inherently long-term nature needed to study WW may have prohibited its study. These differences in rate of WW formation also suggest that the importance of WW formation in tree compartmentalization may vary between trees. For example, for those trees with slow growth rates (eg. Northern temperate hardwood tree species) or for those that produce abundant deposits (eg. latexes and resin), WW formation may not be such an important resistance mechanism against infection. From our studies however, using relatively faster growing eucalypts in a plantation system, the importance of wound wood in tree defence was strongly evident. It would be interesting to compare the wound wood chemistry of eucalypts with that of other woody tree species, which have never been analysed. Such studies may reveal that the extent and effectiveness of WW to successfully compartmentalize injury and infection may vary among tree species and within a species as well as with injury severity and type of infection.

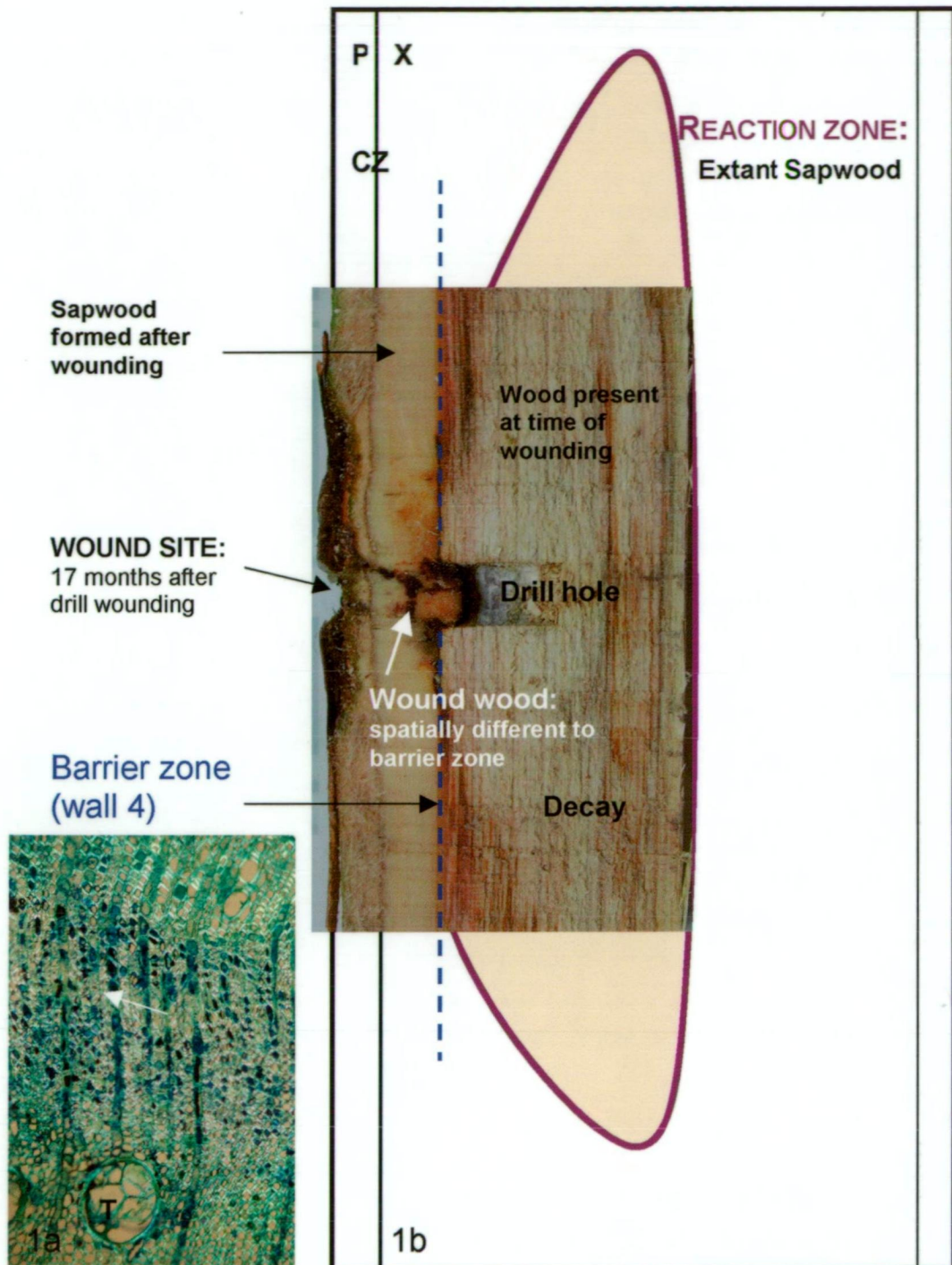


Fig. 9.1 Tree compartmentalization in eucalypts after drill wounding and inoculation with unknown decay fungi a) Transverse section showing typical anatomy of barrier zone comprising of polyphenolic-filled parenchyma (arrow) and tylosis in vessel (T) b) Wound wood formation in *E. globulus* characterized by the presence of dark extractives (arrow).

CHAPTER 10

10.1 SUMMARY

The anatomical and chemical responses of both *E. globulus* and *E. nitens* plantation trees to a variety of mechanical, chemical and biological wound treatments were characterized. Their wound responses were consistently associated with the production of secondary metabolites, macroscopically visible to the naked eye as dark extractives and always located in the new tissue formed after wounding, whether this tissue was;

- a) specialized secretory cells known as kino veins (Chapters 2 and 3)
- b) the less organized wound wood directly adjacent to a wound site (Chapters 4 and 5)
- c) the wound-associated bark of *E. globulus* that developed following green pruning (Chapter 6)
- d) the lesion margin of a stem canker formed in response to natural infection with a *Cytospora* sp. in *E. globulus* (Chapter 7).

Subsequent analysis revealed the complexity of secondary metabolites associated with wound wood and wound-associated phloem. Hydrolyzable tannins, proanthocyanidins, flavonone glycosides, stilbenes, formylated phloroglucinol compounds (FPCs) as well as terpenoids were detected. These compounds were either undetectable in healthy tissue or present at only trace levels. In the case of terpenoids, this class of compounds was largely observed to originate from the traumatic oil glands of wound-associated parenchyma, particularly evident in response to canker infection and green pruning.

From our investigations, the production of secondary metabolites would appear to constitute a major part of the tree defence response in *E. globulus* and *E. nitens*. Crude wound wood extracts have demonstrated *in vitro* antimicrobial activity against decay fungi and gram-positive bacteria as well as *in vitro* antioxidant activity. We proposed that the induction of secondary metabolites at such high levels whether that be in bark or wood, might have a

multi-functional role. The compounds develop as a generic host response to mechanical wounding and not specifically against biotic agents such as fungi and insects. We do not suggest however, that these secondary metabolites do not have an antimicrobial role against those biological agents, particularly as wound wood formation appeared effective at impeding fungal colonization into subsequent functioning sapwood. Admittedly, these findings were based on a small number of samples and further studies must be conducted to confirm our preliminary findings as well as to identify the compound/s responsible for the observed activities. Ultimately, those secondary compounds shown to contribute to tree resistance could then be used as biochemical indicators of tree resistance to search for resistant genotypes, as similarly practiced in other plant systems (examples are given by Heidger and Lieutier, 2002).

10.2 FUTURE WORK

- The influence of different micro-organisms on the quantitative and qualitative chemical composition of wound wood has yet to be examined. Such studies may confirm whether the induction of secondary metabolites in wound-associated tissue is a non-specific host response to wounding or is influenced by the invasion of microbial pathogens.
- The current study investigated the role of secondary metabolites formed in the wound wood and/or wound-associated bark within a host-pathogen system. Future studies should determine the significance of these new findings for other biological interactions including insect-plant interactions. For example, one group of insects that is causing major concern for the New South Wales eucalypt forestry industry are the stem borers. These include the Cerambycids / longicorns (*Phorocantha* spp.) that attack trees mainly via wounds such as cracks in the bark and via branch stubs, as well as the Cossid moths (*Endoxyla cinerea*) that are capable of outright penetration into bark and subsequent sapwood (Angus Carnegie, personal communication). Both

groups of stem borers attack several eucalypt species but in particular, *E. grandis*. The larvae of the Cossid moths apparently feed on the new wound wood formed adjacent to the wound site (Simon Lawson, personal communication). Given our recent improved understanding of the chemistry of wound tissues from this current study, comparing the bark and wound wood chemistry of *E. grandis* with other eucalyptus species may provide a useful system to identify susceptible or resistant species for removal or use in genetic improvement strategies.

- Due to the fortuitous discovery of dark extractives in the lesion margin of *E. globulus* trees naturally infected with a *Cytospora* sp. it was possible to partially characterize the chemical host response to a stem canker infection. Although stem canker infections are currently not a major concern for industry, the rapid expansion of monoculture plantations may lead to increased pest and pathogen damage. An improved understanding of the host mechanisms involved in tree resistance against bark related pathogens will be crucial in finding solutions to this potential problem. This knowledge may also be applied to assist with our understanding of other well-known pathogens such as *Phytophthora* sp. and *Armillaria* sp.
- The discovery of traumatic oil glands in the wound-associated phloem of pruned *E. globulus* was a significant result. Future work will be necessary to understand the biological significance of this finding, particularly in terms of tree defence and its relevance to industry. This study only examined the new tissue formed after first lift pruning. The current pruning prescription for high productivity sites used by the Tasmanian forest industry recommends two to three pruning lifts at ages three to five. If the formation of wound-associated phloem plays a key role in the successful occlusion of pruning wounds, an investigation into the new tissue formed in the last two pruning lifts may provide information on risks of decay associated with pruning at later stages of growth.

- Given that eucalypt wood and bark was shown to contain a complex range of secondary compounds, in addition to providing pulp and timber, the eucalypt industry could supply a commercial source of potentially valuable secondary compounds with pharmaceutical and chemical applications. For example, secondary metabolites produced in eucalypts could be used to combat various human and plant pathogens. FPCs have already reported to show strong antibacterial and antifouling properties (Murata *et al.*, 1990; Takasaki *et al.*, 1994; Singh and Etoh, 1997) while the exogenous application of trans-resveratrol, a compound known to have antifungal character and antioxidant properties, has been shown to improve fruit resistance to fungal infection by acting as a 'natural pesticide' (Urena *et al.*, 2003).

APPENDIX 10.1

Poster Presentations

1. Anatomy and chemistry of wound tissue in *Eucalyptus globulus* and *E. nitens* (2001 -CRC-SPF Annual meeting, Caloundra, Australia).
2. Host responses to natural infection by *Cytospora* sp. in the aerial bark of *Eucalyptus globulus* (2002 - VII Mycological International Congress, Oslo, Norway).
3. Traumatic oil glands induced in the wound-associated phloem of pruned *Eucalyptus globulus* (2002 - VII Mycological International Congress, Oslo, Norway).
4. Eucalypt wound wood extracts show antioxidant and antimicrobial activity (2003 – VIII International Congress of Plant Pathology, Christchurch, New Zealand).

Anatomy and chemistry of wound tissue in *Eucalyptus nitens* and *E. globulus*



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BACKGROUND

The Australian eucalypt forest industry is increasingly applying intensive management practices to produce logs of suitable sawlog quality from plantation eucalypts (Dudzinski *et al.*, 1992; Gerrand *et al.*, 1997). However, practices such as green pruning and thinning result in wounding. These associated xylem injuries are potential source of defect and decay. A better understanding of the tree's defence mechanisms involved in wound repair will provide a framework for developing improved silvicultural practices. Both anatomical and chemical techniques were used to investigate the tree's response to drill and prune wounding treatments.

ANATOMY

DRILL WOUNDING

Macroscopic: Both *E. nitens* and *E. globulus* was found to respond to drill wounding by the production of abnormal tissue and dark extractives referred to as wound tissue (Fig. 1).

Microscopic: Sectioning of wound tissue showed that the tissue consisted mainly of traumatic parenchyma. Staining with various stains indicated the presence of flavan-3-ols (Fig. 2). Some cell walls in the wound tissue were lined with suberin as shown by fluorescence microscopy. Suberin is an impermeable layer often associated with defence response.

PRUNE WOUNDING of *E. globulus*

Macroscopic: Similar wound tissue response was obtained from prune wounding as described above. Freshly cut wound tissue smelt very strongly of essential oils.

Microscopic: Sectioning of phloem wound tissue showed that this tissue contained large clusters of oil glands as indicated by the epithelial cells lining the secretory cavities (Fig. 3).

CHEMISTRY OF WOUND TISSUE BY LC-MS

Analysis of wound tissue by HPLC with ESI-MS revealed the presence of a diverse range of polyphenolic compounds (Fig. 4).

- * hydrolyzable tannins (both gallo- and ellagi-tannins)
- * proanthocyanidins
- * flavanone glycosides e.g. engelitin
- * formylated phloroglucinol compounds (FPCs) (Fig. 5)

(Eyles *et al.*, unpublished)

CHEMISTRY OF WOUND TISSUE BY GC-MS

GC-MS analysis of wound tissue sampled especially from the phloem tissue indicated the presence of a range of terpenes confirming histological findings of oil glands in phloem wound tissue (Fig. 6).

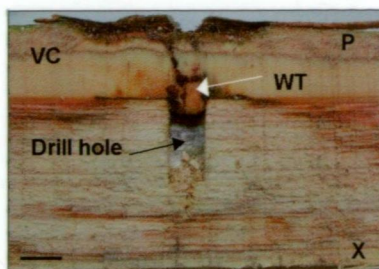


Fig. 1 Production of wound tissue (WT) in response to drill and inoculation treatment in 9-year-old *E. globulus* and *E. nitens*. Scale bar = 1cm. P= phloem, VC= vascular cambium, WT = wound tissue, X= xylem.

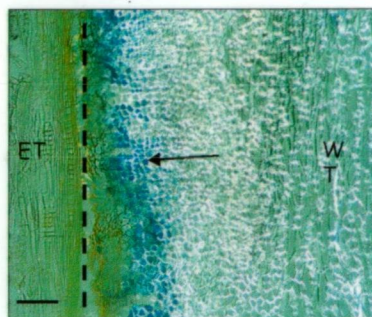


Fig. 2 Longitudinal section of wound tissue stained with DMACA stain testing positive for flavan-3-ols (arrow). ET= extant tissue, WT = wound tissue, Scale bar = 180 µm.

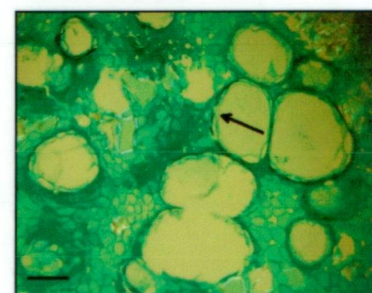


Fig. 3. Longitudinal section of phloem wound tissue containing clusters of oil glands. Note the epithelial cells lining the oil gland (arrow). Scale bar = 133 µm.

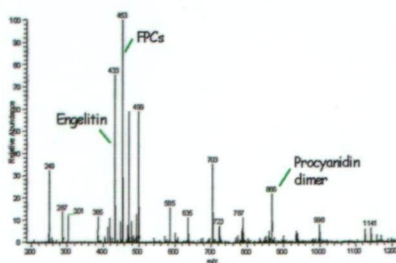


Fig. 4 The summed mass spectra in the range of m/z 180-1200 for wound tissue extracts (70% acetone) of *E. nitens*

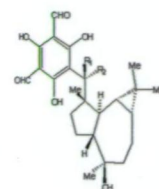


Fig. 5 Structure of two FPCs found in wound tissue: Macrocarpal A ($R_1=H$, R_2 =isobutyl) Macrocarpal B (R_1 =isobutyl, $R_2=H$).

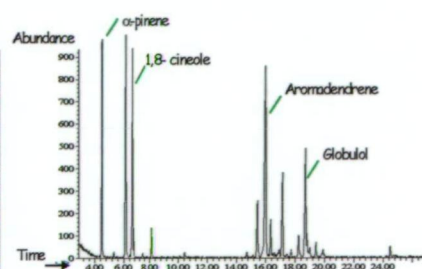


Fig. 6 Typical essential oil composition of oil glands in *E. globulus* phloem wound tissue by GC-MS

BIOLOGICAL SIGNIFICANCE OF WOUND TISSUE?

Antimicrobial role? Preliminary antifungal and antibacterial bioassays suggest the wound tissue contain extractives that may have an antimicrobial role. The wound tissue does appear to restrict the fungal infection into the new tissue (Fig. 1).

Antioxidant role? Stresses such as wounding are known to are found to generate production of free radicals that damage plant DNA, proteins and membranes (van Doorslaer *et al.*, 1999). There is increasing evidence to suggest that polyphenolic compounds such as those found in wound tissue may function as antioxidants (Hagerman, 1998).

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Traumatic oil glands in the wound-associated phloem of pruned *Eucalyptus globulus*

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PRUNE WOUNDING

- Trees respond to prune wounding by the formation of both biochemical and anatomical boundaries to compartmentalize wounded area (1).
- New tissue formed immediately adjacent to the wounding site has received little attention in either hardwoods or softwoods.
- As well as compartmentalizing infections on the inside, trees also need to close wounds on the outside to seal entry points for decay. In the case of pruning wounds, rapid and clean occlusion of pruned stubs would appear to be essential to reduce risk of decay entry.

NEW DISCOVERY OF TRAUMATIC OIL GLANDS IN EUCALYPTS

- In the two years following pruning, complete occlusion of the pruned branch had been achieved in 5 year-old *E. globulus* (Fig. 1A).



Fig. 1 Longitudinal (radial) micrograph of new phloem formed 2 years after pruning in *E. globulus*

- The new phloem formed after wounding was predominately composed of irregularly shaped secretory cavities often distributed in clusters within the traumatic parenchyma (Fig. 2A). These secretory cavities were lined with distinct epithelial cells suggesting these structures were oil glands (Fig. 2B).

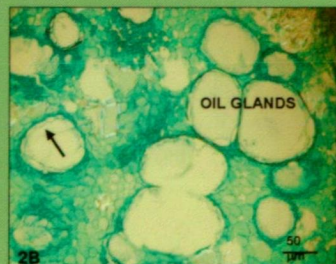


Fig. 2A Enlarged area of square in Fig. 1 showing prolific production of oil glands seen at low magnification and at higher magnification (2B) showing cluster of oil glands with epithelial cells (arrow). Stain: toluidine blue.

GC-MS ANALYSIS CONFIRMS PRESENCE OF TWO TERPENE COMPOSITIONS

- Wound-associated phloem was found to contain significantly greater amounts (> 50 times) of total oil than healthy phloem.
- Two distinct phenotypic oil compositions were detected a) 'α-phellandrene' type b) 'α-pinene' type which contained substantial amounts of α-pinene and 1,8-cineole but no α-phellandrene (Table 1).

Table 1. Two 'chemotypes' detected in wound-associated phloem extracts of pruned 5-year-old *E. globulus* by GC-MS.

No.	Oil compounds	α-PHELLANDRENE TYPE	α-PINENE TYPE
1	α-pinene	10.8 ± 0.4	20.9 ± 1.6
2	α-phellandrene	24.4 ± 1.7	n.d
3	myrcene	0.6 ± 0.1	n.d
3	p-cymene	2.2 ± 0.8	0.6 ± 0.6
4	1,8-cineole	6.5 ± 0.6	18.7 ± 0.7
5	limonene	1.1 ± 0.1	2.1 ± 0.03
6	γ-terpinene	0.4 ± 0.1	1.3 ± 0.6
7	terpinolene	1.0 ± 0.2	0.4 ± 0.2
8	terpinyl acetate	n.d	1.7 ± 0.9

OIL COMPOUNDS DETECTED IN BOTH CHEMOTYPES

9	α-gurjunene	5.2 ± 0.4
10	β-gurjunene	1.1 ± 0.1
11	aromadendrene	16.0 ± 0.8
12	calamenene	0.7 ± 0.05
13	alloaromadendrene	2.7 ± 0.06
14	unknown sesquiterpene hydrocarbon	0.6 ± 0.02
15	viridiflorene	6.0 ± 0.2
16	d-cadinene	0.4 ± 0.02
17	ledol	1.8 ± 0.07
18	globulol	7.8 ± 0.3
19	viridiflorol	1.4 ± 0.03
20	unknown sesquiterpene alcohol	0.7 ± 0.04
21	rosifolol	0.8 ± 0.02
22	β-eudesmol	0.8 ± 0.02
23	unknown sesquiterpene alcohol	1.1 ± 0.1
24	unknown volatile phenol M.W. 252	1.2 ± 0.2
25	unknown volatile phenol M.W. 248	0.9 ± 0.1
26	Phenol M.W. 252	1.1 ± 0.2

n.d. = not detected

TRAUMATIC OIL GLANDS INVOLVED IN DEFENCE???

- Healthy phloem provides an important structural and chemical barrier against insect and/or fungal pathogens.
- The wound-associated phloem contained a variety of monoterpenes including α-pinene. α-pinene has been found to exhibit strong biological activity towards pathogenic fungi and bark beetles (2, 3).
- We suggest that the concentrated amounts of terpenes produced in the wound-associated phloem of eucalypts may have a similar protective function as that shown for the terpenes produced in the resin ducts of many conifers. Further detailed studies will be necessary before such conclusions can be drawn.

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Host responses to natural infection by a stem canker (*Cytospora* sp.) in the aerial bark of *Eucalyptus globulus*: a preliminary report



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EUCALYPT LESION MARGIN (LM) FORMED IN RESPONSE TO STEM CANKER INFECTION: DIFFERENT FROM OTHER WOODY SPECIES

- For the first time, the anatomical and chemical responses to natural infection by a stem canker fungus (*Cytospora* sp.) in the aerial bark of 3-year-old *Eucalyptus globulus* (Fig. 1) sampled from a Tasmanian plantation is presented.
- LM was characterized by layer of dark extractives (Fig. 2a & 3a)
- LM of severe canker infections contained clusters of traumatic oil glands (Fig. 3).
- Some LMs of superficial canker infections (3a) were observed to contain a complete and continuous wound periderm (WP) comprising of a single layer of suberized thin-walled phellem cells followed by up to 11 layers of ligno-suberized thick-walled phellem cells (Fig. 3b & 3c). Other LMs were characterized by an incomplete and discontinuous WP (Fig. 3d).
- ALL LMs were associated with new parenchyma that stained strongly for the presence of flavan-3-ols in their cell lumens with DMACA histochemical stain. Hereafter referred to as 'induced DMACA parenchyma' (Fig. 3c & 3d), these cells were formed prior to the formation of the WP.
- Chemical analysis of LM extracts by GC-MS, HPLC-UV and HPLC-ESI-MS revealed presence of a diverse range of secondary metabolites (e.g. FPCs Fig. 4). These compounds were undetectable in healthy tissue or else present at significantly lower concentrations than in the LM ($P < 0.05$) (Table 1).

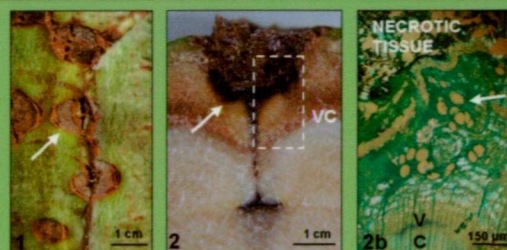


Fig. 1. Necrotic lesion in aerial bark (arrow). Fig. 2a. LM of severe canker infection (transverse). Note layer of dark extractives (arrow). 2b. Enlargement of square in 2a. Note presence of traumatic oil glands (arrow). Stain: toluidine blue, VC, vascular cambium.

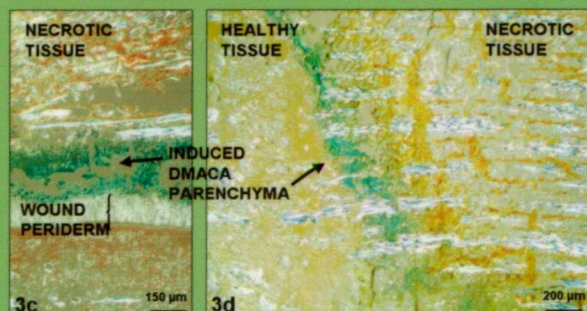
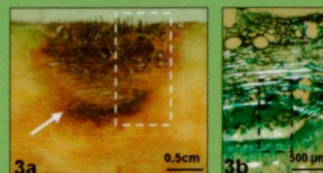


Fig. 3a. LM of superficial canker infection. Also note layer of dark extractives (arrow). 3b. Enlargement of square in 3a showing WP. Stain: toluidine blue. 3c. Enlargement of square in 3b. Continuous WP comprising of thick-walled phellem cells and induced DMACA parenchyma (arrow). Stain: DMACA. 3d. LM of discontinuous and incomplete WP. Stain: DMACA.

Table 1. Concentration (% wet weight) of 5 compound classes detected between 3 tissue types.

Secondary compounds sapwood	Lesion margin	Healthy phloem	Healthy
◆ Catechin (flavan-3-ol)	0.53 ± 0.06	0.07 ± 0.01	0.02 ± 0.008
◆ Engletin (flavonoid glycoside)	0.04 ± 0.019	n.d.	n.d.
◆ FPCs (formylated phloroglucinol compounds)	0.64 ± 0.17	0.14 ± 0.07	n.d.
◆ Pedunculagin (hydrolyzable tannin)	5.84 ± 1.20	0.38 ± 0.11	0.067 ± 0.019
◆ Polymeric PAs (proanthocyanidins)	16.47 ± 1.28	2.69 ± 0.31	n.d.
◆ Terpenes	0.89 ± 0.2	0.23 ± 0.002	n.d.

n.d. = not detected

EFFECTIVE BARRIER AGAINST STEM CANKER INFECTION???

- Host response to wounding and attack by fungal pathogens includes a combination of chemical and physical barriers in an attempt to re-establish tissue integrity and aid in defence (1, 2). The chemical and physical barriers of the eucalypt lesion margin would appear to be effective at confining the spread of the weak pathogen (*Cytospora* sp.) in *E. globulus*. Future work may involve studying the sequence of events leading to LM formation following controlled artificial inoculations with other canker causing fungi paying particular attention to formation of induced DMACA parenchyma.

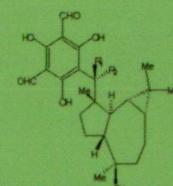


Fig. 4 Two FPCs found in LM: Macrocarpal A ($R_1=H$, $R_2=$ isobutyl) Macrocarpal B ($R_1=$ isobutyl, $R_2=H$).

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***Eucalyptus* wound wood extractives show antimicrobial and antioxidant activity (A case study with *E. globulus* and *E. nitens*)**

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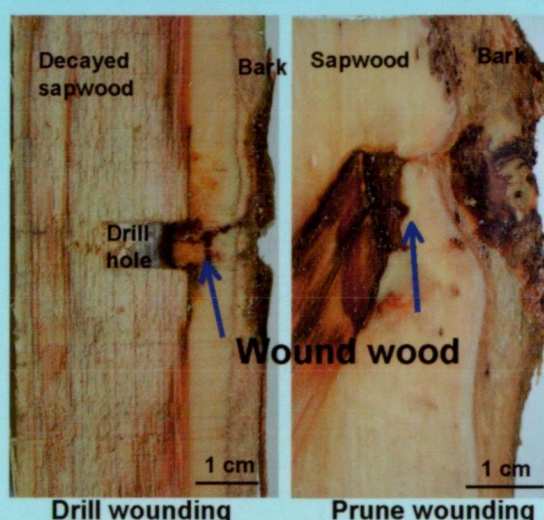
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Eucalypt wound wood was characterized by dark extractives, regardless of injury type

In two commercially important hardwood species, *Eucalyptus globulus* and *E. nitens*, the new tissue formed adjacent to the wounding site (referred to as 'wound wood' and characterized by dark extractives) appeared to impede fungal colonization¹.



Eucalypt wound wood was composed of a complex range of secondary metabolites

❖ Analysis of wound wood extracts by HPLC-UV, HPLC-ESI-MS and GC-MS identified the presence of various polyphenols, terpenoids and formylated phloroglucinol compounds (FPCs)^{2,3}.

We suggest wound wood extractives have both an antimicrobial and antioxidant role

- ❖ *In vitro* antifungal and antibacterial assays show extractives possess strong antimicrobial activity.
- ❖ *In vitro* antioxidant assays show that some compounds identified in wound wood extracts have antioxidant activity.

Evidence for antioxidant role

*Superoxide dismutase (SODase)-like antioxidant activity of compounds and fractions isolated from *E. globulus* wound wood extracts*

Compound/fraction inhibition** 11.9 µg/ml	IC ₅₀ *	% (µg/ml)
engletin	0.0	0.47
penduculagin	5.6	82
original wound wood	5.6	76
methanol I PA [#]	7.2	70
methanol/acetone PA	4.7	81
methanol II PA	13.0	48
gallic acid [^]	6.0	97
pentagalloyl-glucose [^]	<4.7	90

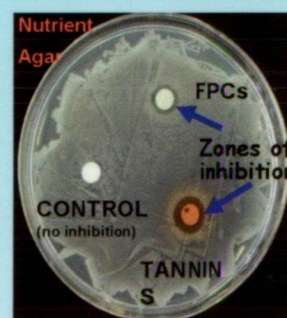
*IC₅₀ = sample concentration to obtain 50% inhibition

** % inhibition using 0.25 µg/ml of sample

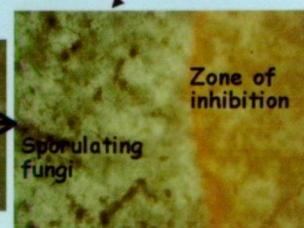
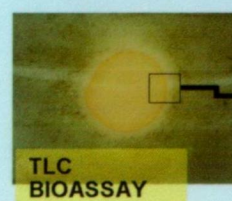
[#] PA = proanthocyanidins

[^] standard (identified in sample)

Evidence for antimicrobial role



Strong activity shown against Gram positive bacteria eg. *Bacillus subtilis* and fungi eg. *Cladosporium cladosporioides*



The complex range of secondary metabolites in wound wood suggests that for eucalypts, wound wood formation is an important resistance mechanism in compartmentalizing infection and/ or injury¹⁻⁶

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